

Digitized by the Internet Archive
in 2017 with funding from
University of Alberta Libraries

<https://archive.org/details/glowach1970>

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

QUANTITATIVE ASSAY OF ANTI-FERTILITY AGENTS

The undersigned certify that they have read, and recommend
to the Faculty of Graduate Studies for acceptance, a thesis entitled
by

"Quantitative Assay of Anti-Fertility Agents" submitted by Sharon
Joanne Glowach in partial fulfillment of the requirements for the
degree of Master of Science.

SHARON JOANNE GLOWACH, B.Sc.

A THESIS

L. S. G. G. G.
Supervisor

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF MASTER OF SCIENCE

R. L. G. G.

Byron K. G. G.

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

Date July 25/70

EDMONTON, ALBERTA

FALL, 1970

ABSTRACT

An investigation was undertaken to quantitatively assay the synthetic steroid components of the oral anti-fertility preparations. Methods were developed which utilized either infrared spectrophotometric or gas-liquid chromatographic analytical procedures.

In the IR analysis linear calibration curves were obtained for all the pure steroids. When the technique was applied to commercially-available oral contraceptives, the majority of the component progestogens were readily and accurately estimated. The estrogen component, however, was present in a concentration which was too low for quantitative infrared analysis to be feasible.

Gas-liquid chromatography was chosen as a correlative to the infrared method due to its greater analytical sensitivity. Through this procedure an attempt was made to quantitatively measure both the estrogen and progestogen components as they are found in pharmaceutical dosage forms. Linear, but not constantly reproducible calibration curves were obtained on a 3 per cent OV-1 column for several of the pure estrogen-progestogen combinations. When the developed method was applied to a number of trade preparations, all calculated percentage recoveries varied widely from the claimed potency. The possible reasons for these discrepancies are discussed.

Week); Syntex Limited (norethindrone acetate, Norinyl [®] 1 mg., 2 mg., and Norquon [®]); and John Wyeth and Brother (Canada) Limited (d-Norgestrel (as dl-racemate) and Ovral [®]).

Sincere thanks are also extended to Mrs. Gail Conway for her capable assistance in the typing of this manuscript.

ACKNOWLEDGEMENTS

The author gratefully thanks Dr. L. G. Chatten and Dr. E. J. Triggs for their valuable assistance, encouragement, and criticism given throughout the course of this study.

Acknowledgements are also made to the Faculty of Pharmacy and Pharmaceutical Sciences for financial support given to the author in the form of teaching assistantships, and the Medical Research Council of Canada for financial aid in the form of a studentship.

In addition, the writer greatly appreciates the pure reference materials, pharmaceutical dosage forms, and pertinent information supplied by the manufacturing companies before and during the course of the research project. These pharmaceutical manufacturers include the following: Delmar Chemicals Limited (ethinyl estradiol, USP); Eli Lilly and Company (Canada) Limited (C-Quens[®]); Mead Johnson (Canada) Limited (megestrol acetate, mestranol, norethindrone, dimethisterone, Oracon[®], and Ovex[®]); Ortho Pharmaceutical (Canada) Limited (norethindrone, Ortho-Novum[®] 1 mg., 2 mg., 5 mg., and SQ); Parke, Davis and Company, Limited (Norlestrin[®] 1.0 mg., and 2.5 mg.); Roussel (Canada) Limited (ethinyl estradiol USP); G. D. Searle and Company of Canada, Limited (mestranol, ethynodiol diacetate, norethynodrel, Enovid[®] 5 mg., 10 mg., Enovid-E[®], Ovulen[®] 0.5 mg., and Ovulen[®] Three Week); Syntex Limited (norethindrone acetate, Norinyl[®] 1 mg., 2 mg., and Norquen[®]); and John Wyeth and Brother (Canada) Limited (d-Norgestrel (as dl-racemate) and Ovrall[®]).

Sincere thanks are also extended to Mrs. Gail Conway for her capable assistance in the typing of this manuscript.

TO

ALLAN MICHAEL, my son

AND

JOSEPH, my husband

TABLE OF CONTENTS

	Page
Abstract	iii
Acknowledgements	iv
Dedication	v
List of Tables	viii
List of Figures	x
Introduction	1
Statement of the Problem	33
Experimental	34
I. Infrared Spectrophotometry	34
Apparatus	34
Chemicals	34
Reagents	34
Reference Standards	34
Procedures	35
A. Siliconization of Glassware	35
B. Infrared Spectra of Probable Tablet Excipients	35
C. Determination of an Absorption Peak Most Suitable for Quantitative Analysis	36
D. Preparation of Calibration Curves	37
E. Quantitative Assay of Pharmaceutical Preparations	45
II. Gas-liquid Chromatography	50
Apparatus	50
Column	50
Reagent	50
Internal Standard	50

TABLE OF CONTENTS - Continued	Page
Reference Standards	50
Procedures	51
A. Siliconization of Glassware	51
B. Internal Standard (Cholestane) Solution	51
C. Preparation of Calibration Curves	51
D. Quantitative Assay of Pharmaceutical Preparations	59
Results and Discussion	63
I. Infrared Spectrophotometry	63
Theory	63
Quantitative Analysis	69
Calibration Curves	72
Assay of Pharmaceutical Dosage Forms	72
II. Gas-liquid Chromatography	97
Theory	97
Quantitative Analysis	98
Calibration Method	99
Assay of Pharmaceutical Dosage Forms	101
Summary and Conclusions	116
Bibliography	121
Appendix	128

LIST OF TABLES

<u>Table</u>		<u>Page</u>
I	Determined Refractive Index and Ultraviolet Values for Oral Contraceptive Steroids	15
II	Determined Ultraviolet Values for Progestogens	18
III	Determined Ultraviolet Values for Estrogens	19
IV	Synthetic Estrogens Currently Available as Oral Contraceptives in Canada	29
V	Synthetic Progestogens Currently Available as Oral Contraceptives in Canada	30
	Trade Preparations Assayed Through Infrared Spectrophotometry	
VI	A. Combination Tablets	47
VII	B. Sequential Tablets	49
VIII	Trade Preparations Assayed Through Gas-liquid Chromatography	62
IX	Absorption Peak Area Selected for Quantitative Analysis	64
	Infrared Assay of Pharmaceutical Dosage Forms	
X	A. Combination Tablets	84
XI	B. Sequential Tablets	85
XII	Assay of Pharmaceutical Dosage Forms (GLC) A. Combination Tablets B. Sequential Tablets	111
XIII	Data for the Determination of Calibration Curves (IR) Estrogens Progestogens	128
	Assay of Pharmaceutical Dosage Forms (IR)	
XIV	A. Combination Tablets	131
XV	B. Sequential Tablets	133

LIST OF TABLES - Continued

<u>Table</u>	<u>Page</u>
XVI Data for the Preparation of Calibration Curves (GLC)	
A. Chlormadinone Acetate and Mestranol	134
B. Ethynodiol Diacetate and Mestranol	135
C. Norethynodrel and Mestranol	
(i) First Determination	136
(ii) Second Determination	137
XVII Assay of Pharmaceutical Dosage Forms (GLC)	
A. Combination Tablets	138
B. Sequential Tablets	139

LIST OF FIGURES

<u>Figure</u>		<u>Page</u>
I.	<u>Infrared Spectrophotometry</u>	
1	Wavelength Selected for the Analysis of the Pure Steroid Estrogens	67
2	Wavelength Selected for the Analysis of the Pure Steroid Progestogens	68
3	A Typical Representative Peak Selected for Quantitative Infrared Analysis	71
4	Calibration Curve for Ethinyl Estradiol in Chloroform	73
5	Calibration Curve for Mestranol in Chloroform	74
6	Calibration Curve for Chlormadinone Acetate in Chloroform	75
7	Calibration Curve for Dimethisterone in Chloroform	76
8	Calibration Curve for Ethynodiol Diacetate in Chloroform	77
9	Calibration Curve for Megestrol Acetate in Chloroform	78
10	Calibration Curve for Norethindrone in Chloroform	79
11	Calibration Curve for Norethindrone Acetate in Chloroform	80
12	Calibration Curve for Norethynodrel in Chloroform	81
13	Calibration Curve for d-Norgestrel (as <u>dl</u> -racemate) in Chloroform	82
14	One Example of an Oral Contraceptive Preparation whose Diluents do not Interfere with Infrared Analysis in the Area Selected	86
15	One Example of an Oral Contraceptive Preparation whose Diluents Interfere with Infrared Analysis in the Area Selected	87
16	Example of an Attempt to Analyze the Estrogen Component of a Trade Preparation	89
17	Variation in the Infrared Spectra of C-Quens [®]	91

LIST OF FIGURES - Continued

<u>Figure</u>		<u>Page</u>
18	Method of Measuring Transmission in the Pharmaceutical Preparations	92
19	Variation in the Infrared Spectra of Ovulen®	94
II. <u>Gas-liquid Chromatography</u>		
20	Calibration Curve for Chlormadinone Acetate and Mestranol in Chloroform	102
21	Calibration Curve for Ethynodiol Diacetate and Mestranol in Chloroform	103
22a	Calibration Curve for Mestranol from the Norethynodrel and Mestranol Solution Mixtures	104
22b	Calibration Curve for Norethynodrel from the Norethynodrel and Mestranol Solution Mixtures	105
23	A Typical Chromatogram Obtained for Chlormadinone Acetate 6.0 $\mu\text{g.}/2\mu\text{l.}$, Mestranol 3.0 $\mu\text{g.}/2\mu\text{l.}$, and Cholestane 5.0 $\mu\text{g.}/2\mu\text{l.}$	106
24	A Typical Chromatogram Obtained for Ethynodiol Diacetate 6.0 $\mu\text{g.}/2\mu\text{l.}$, Mestranol 3.0 $\mu\text{g.}/2\mu\text{l.}$, and Cholestane 5.0 $\mu\text{g.}/2\mu\text{l.}$	107
25	A Typical Chromatogram Obtained for Norethynodrel 12.0 $\mu\text{g.}/2\mu\text{l.}$, Mestranol 6.0 $\mu\text{g.}/2\mu\text{l.}$, and Cholestane 5.0 $\mu\text{g.}/2\mu\text{l.}$	108
26	An Illustration of the Retention Time, Shape of Peaks, and Overlapping of Peaks Obtained for Norethindrone 10.0 $\mu\text{g.}/2\mu\text{l.}$, Mestranol 5.0 $\mu\text{g.}/$ 2 $\mu\text{l.}$, and Cholestane 5.0 $\mu\text{g.}/2\mu\text{l.}$	109

INTRODUCTION

1. HISTORICAL

The first known medical text on contraception was an Egyptian papyrus dating from about 1850 B.C.. It advocated two methods — the use of crocodile dung mixed with a vehicle to form a pessary for insertion into the vagina, and irrigation of the vagina with honey and a native sodium carbonate. Both of these procedures are now recognized as having caused weak spermicidal action, the honey also reducing sperm motility. Early history involving other civilizations also mentions frequently the use of a sticky substance, such as honey, for the same purpose.

Among the Hebrews a religious duty of the male was to obey the commandment of propagation. No such rule affected the female who accepted the responsibility of the prevention of pregnancy. There have been references in the Bible and the Talmud where the ancient Jewish people allowed women to "take the cup of roots" to render them sterile. A description of "the root" by the first century A. D. Greek historian, Dioscorides, bears a resemblance to the Mexican yam, a major plant source for the synthesis of present day steroid hormones.

Aristotle produced the first known Greek reference to contraception in his writings, describing the "anointing of that part of the womb where the seeds fall with oil of cedar, or with ointment of lead, or with frankincense mixed with olive oil". He believed that the smoothness of the mixture prevented conception, but it was the oil which reduced motility of spermatozoa.

Dioscorides' "Medical Herbal" described four contraceptive methods.

1. substances causing sterility

- a) a drink made of the fruit of the Chaste Tree plus wine
- b) the leaves of a willow, beaten and taken in water
- c) a drink composed of the rennet of a hare

2. medicated pessaries

- a) pepper, alum, and various herbs placed in the mouth of the womb
- b) tips of acacia triturated with honey used to moisten lint which was subsequently used as a tampon

3. sticky substances used to anoint the genitals

- a) cedar gum
- b) alum

4. amulets

- a) asparagus hung in a container about the neck

The Roman gynecologist, Soranos, who practised medicine during the reigns of Trajan and Hadrian (98-138 A.D.), described the most brilliant and original prophylactic techniques prior to the nineteenth century. He was the first to rationalize that there were certain periods during a female's cycle which were suitable for conception and that it was safer to prevent pregnancy than to destroy the fetus. Soranos was able to distinguish between contraceptives and abortifacients and mentioned both indications and contraindications for their use.

Islamic medicine predominated for about the next nine hundred years. Skilled Arabs introduced many new medical and surgical

techniques but the methods of contraception and abortion were patterned according to Greek influence. Even though the writings of Hippocrates, Galen, the Jewish doctors, and the brilliant Persian "Canon of Medicine" were available during the Medieval period in Europe, the practice of general medicine declined. This eventually led to the use of irrational methods, such as magic formulae and numbers. Bezoar stones taken from the stomachs of goats and gazelles and worn in fancy lockets as a cure-all, spitting three times into the mouth of a frog, or eating bees were other modes of the time.

The initial report on the discovery of the condom was by the Italian anatomist, Fallopius, in 1564. It was originally made of linen and intended to guard against syphilis. When this was later extended to thin animal membranes its contraceptive use became apparent. The origin of the name is unknown but one of the more prevalent theories is that it was named after a man called Condom who was a doctor at the court of Charles II of England. Charles, being responsible for a large number of illegitimate children, was so delighted with this expedient that he conferred a knighthood upon the physician. Such notoriety came to the medical man that he was forced to change his identity.

Both the name and the device became well known very rapidly, followed by the appearance of both "original and used" condom shops across Europe by 1701.

Development of spermicides really began with the work of Anthony van Leeuwenhoek when in 1678, he observed "sperm" in canine semen and found that dilution with rain water decreased their motility. When he changed the pH of semen solutions through the

addition of vinegar or sodium hydroxide, he found sperm in acid media to be inactivated but activity of immobile sperm to increase upon addition of alkali.

By the early 1800's, a movement of English propagandists campaigned to educate and inform the masses about family limitation with its social and economic advantages. The theory was that population tends to multiply faster than the means of subsistence, and the adjustment of overpopulation to food supply is accompanied by vice and misery.

From about 1908 to the late 1920's a wide range of caps and diaphragms were mass produced. Rubber, metal, cellulose, and other firm substances were used for cervical caps. Other types intended for use with a chemical were made from spongy materials.

About this same time the first commercial spermicides appeared. These included alkalis, acids, inorganic salts, organic antiseptics, organic reducing agents and alkaloids. Further study of chemical contraception ultimately led to the modern use of foam jellies.

The vulcanization of rubber in 1843 made a great difference in the use of the condom. With the development of a more reliable and less expensive product, skin condoms dropped from popularity. In the early 1930's liquid latex replaced crepe rubber with the added advantage that the evenly coated latex prevented the formation of a drop at the end of the condom.

With the introduction of the intra-cervical device about the end of the nineteenth century, almost universal medical condemnation arose with regard to this procedure. This device consisted of a metal stem

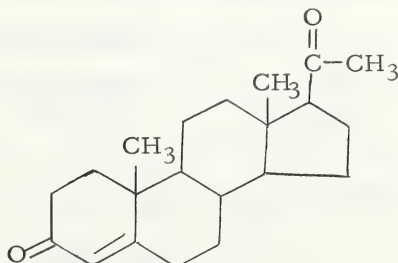
fixed to a wide disc or circle to straighten a crooked womb. Soon it was evident that the conception rate of individuals using this apparatus was reduced.

Before long the first intra-uterine catgut loop appeared. It was inserted entirely into the body of the uterus for the sole purpose of contraception. Later developments included silkworm gut and, in 1959, polyethylene was the substance of choice. This material provides a major advantage in comparison with other methods in that it can be pulled out straight to fit into a tube, and when released after insertion, it moves back into its original shape. The conception rate of females using this device is about two to three per cent, but for certain personal or for medical reasons this procedure is unacceptable to many individuals.

Over the ages, a constant search has continued to find an effective oral contraceptive which would fulfill the basic requirements of efficacy, acceptability, safety, ease of administration, moderate cost and common availability.

During the 1880's scientists were beginning to suspect that the ovaries secreted a substance which inhibited ovulation, but it was not until 1929 that Corner and Allen isolated the first ovarian hormone, progesterone. In 1934 its structure was established as

Progesterone

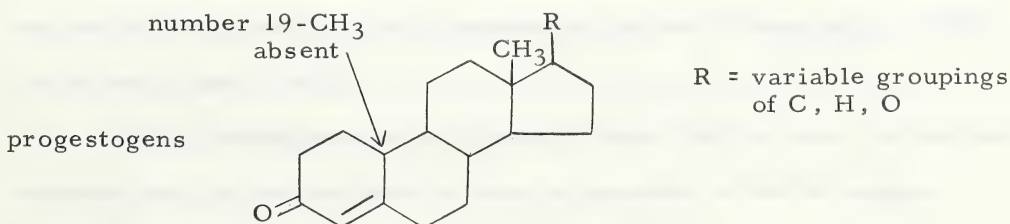


It soon became known that this hormone had the power to inhibit ovulation. However, it was not until the early 1950's that two different research groups looking for answers to seemingly opposite problems in fertility put this knowledge to use in contraception. Pincus and his colleagues were looking for a practical way to prevent conception and Rock and his associates were trying to help women who were apparently infertile. It was found that even though progesterone did inhibit ovulation, large and frequent doses were necessary orally, and if given by injection the treatment was painful.

Women who were unable to conceive and were subsequently given massive doses of the hormone for three months showed many symptoms compatible with pregnancy, but not pregnancy itself. When treatment was discontinued the menstrual cycles became normal and a large percentage of the women became pregnant. Doctors Rock and Pincus began to share experiences and as a result administered progesterone in twenty-day cycles. The major drawback was that progesterone was expensive and was required in such large doses that it was impractical for general use.

Searle Laboratories initiated the research into orally-active compounds structurally similar to progesterone (Colton, 1955).

These original progestogens (19-nor testosterone) differed from the natural steroids in that they lacked a β -methyl side chain in the number 19 position.



However , later progestogens included those with a 19-methyl group.

After intensive rat and rabbit tests , a new synthetic steroid, norethynodrel, was found to be at least ten times as active orally as progesterone. Commencement of clinical trials in 1956 proved norethynodrel to be 100 per cent effective in inhibiting ovulation (Pincus , 1956; Pincus et al. , 1956; Rock et al. , 1956; Rock et al. , 1957).

The potentialities of this compound became apparent and the demand for this type of product rapidly expanded research in this area. Countless synthetic contraceptive steroids were developed and tested (Hertz et al. , 1956; Greenblatt, 1956; Colton et al. , 1957; Tyler and Olson, 1959; Iriarte et al. , 1959; David et al. , 1959; Ringold et al. , 1959; Edgren et al. , 1963; Mears , 1964).

The possibility that norethynodrel and norethindrone possessed oral contraceptive properties first appeared in the scientific literature in 1956; while similar properties were reported for ethinyl estradiol and mestranol in 1957; for norethindrone acetate, megestrol acetate, chlormadinone acetate, and dimethisterone in 1959; and for d-norgestrel (as dl-racemate) and ethynodiol diacetate in 1963.

Several of the earliest orally active progestogens used in inhibiting ovulation were later found to be contaminated with estrogen. This proved to be a fortunate accident , however , for it served to show that the estrogen enhanced the suppressive effect of the progestogen and led to the general use of a mixture of the two (Pincus et al. , 1958; Swyer and Little , 1962).

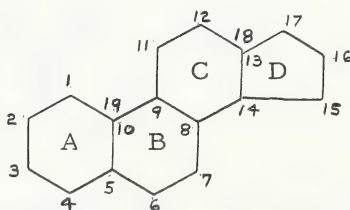
Early investigations had already shown that the ovary of various mammalian species secreted several substances — the estrogens ,

progesterone, and androgens.

It is now recognized that, in addition, the ovary secretes relaxin (a nonsteroid hormone) and perhaps adrenal corticosteroids, as well. Estrogens and progesterone are also produced by the placenta, adrenal gland, and by the testes.

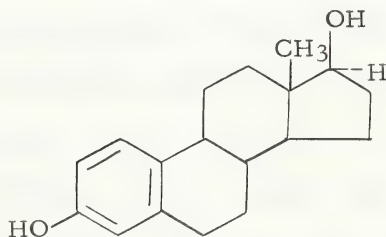
All natural estrogens and progesterone are steroids; their chemical nucleus is the hydrocarbon perhydrocyclopentanophenanthrene.

perhydrocyclopentano-
phenanthrene nucleus



Of the three main estrogens of man, the most potent is the ovarian-produced 17β -estradiol.

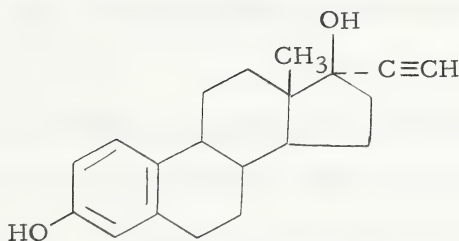
17β -Estradiol



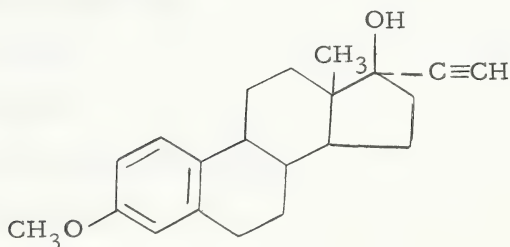
Natural estrogens are unique among steroids in that the ring A of the nucleus is both unsaturated and contains a phenolic group in position 3.

Since 17β -estradiol is not effective orally in small doses, the economic problems of obtaining large quantities of the product led to the synthesis of the orally-effective estrogenic steroids, ethinyl estradiol and mestranol.

Ethinyl Estradiol



Mestranol



Chemical alteration of the number 17 α -hydrogen in 17 β -estradiol to acetylene in ethinyl estradiol provides protection from inactivation by the liver and makes this oral synthetic steroid the most active estrogen known. The 3-methyl ether of ethinyl estradiol, mestranol, is also highly active and is widely used in combinations of oral contraceptives.

When a major quantity of progestogen plus a minor quantity of estrogen are combined to form the original "combination" oral contraceptive, effective conception control results. However, the product is useless to many women because it produces severe gastrointestinal side effects, especially at the beginning of treatment. The high incidence of nausea and vomiting prompted the development of the "sequential" method. This technique involves the use of an estrogen only for fifteen or sixteen days of the cycle, followed by an estrogen-progestogen combination for the following five or six days. The rationale is that this follows more closely the natural hormonal

balance of the female cycle , thereby reducing gastrointestinal side effects .

Even though the synthetic estrogens and progestogens are most widely known for their contraceptive effect , they are also used alone or in combination for the diagnosis and treatment of various functional disorders including:

1. Menopause
 - a. natural
 - b. artificially-induced
2. Amenorrhea
 - a. primary
 - b. secondary
3. Abnormal functional uterine bleeding
4. Dysmenorrhea
5. Premenstrual tension
6. Suppression of lactation
7. Threatened and habitual abortion
8. Infertility
9. Breast cancer
10. Diabetes in pregnancy
11. Osteoporosis
12. Failure of ovarian development
13. Acne
14. Hirsutism
15. Prevention of heart attacks
16. Endometriosis
17. Evaluation of ovarian function and diagnosis of pregnancy

2. ANALYTICAL

The advent of the commercial oral contraceptive and its subsequent utilization by millions of the female population necessitated the development of accurate measurements for component estrogenic and progestational steroids. The problem confronting the analyst is fundamentally one of ensuring that the dispensed medicament contains the labelled ingredients and conforms with the quantitative declaration.

Because of the extremely close chemical relationship of members within each class of compound, the method of quantitative assessment should also be one of identification and differentiation. In those tablets containing both estrogen and progestogen, the method applied must enable the minor estrogenic constituent to be accurately determined. Lastly, any method devised should utilize a minimum number of oral contraceptive tablets for an individual assay.

Various colorimetric methods, used primarily in the determination of estrogens in biological fluids, were developed long before the commencement of clinical trials on synthetic progestogens.

Gibbs (1927) reported the use of 2,6-dibromoquinone chlorimide. In 1931, Kober introduced a sulfuric acid-phenol-iron reagent which gave a violet color on reaction with estrogens. Other methods reported were based on the coupling with diazotized p-nitroaniline (Schmulowitz and Wylie, 1935), with diazotized sulfanilic acid (Talbot et al., 1940; Bender and Wilson, 1947) and its derivatives (Mitchell and Davies, 1954), and with tetrazotized dianisidine (Liebermann, 1952).

More recently Heusghem and Jehotte (1957) determined ethinyl estradiol in pharmaceutical preparations by treatment with acetic

anhydride and by the addition of an ethanol:sulfuric acid (20:80) reagent. An intense rose color with green fluorescence is formed but, unfortunately, the resulting rapid fading is detrimental to the precision and accuracy of the method. Gänshirt and Polderman (1964) developed a chromogenic species with mestranol using an aqueous sulfuric acid reagent; Shroff and Huettemann (1967) employed a phenol-sulfuric acid reagent to measure mestranol. Study of these reactions led to the development of a methanol-sulfuric acid chromogenic reagent of definite and critical proportions which provided a stable and intense color suitable for measurement of microgram amounts of estrogens (Tsilifonis and Chafetz, 1967). The reaction was found to be surprisingly specific for ethinyl estradiol and its 3-ethers from among a number of representative estrogens. A later publication by Eli Lilly and Company indicated that this reagent has been used for several years in routine control assays of mestranol (Comer et al., 1968).

Comer also extended the use of sulfuric acid:methanol (70:30) reagent to the assay of unit tablets of mestranol. Optimum precision and accuracy was obtained by direct dissolution of sample tablets and standardized reference tablets in the reagent. An alternate method utilizing the basic automatic analyzer (Auto Analyzer) and a fluorometer was more rapid but less precise than the direct method. Nitrate, nitrite, and hydrogen peroxide were found to retard the reaction with sulfuric acid.

A protraction of the automated colorimetric analysis was applied to ethinyl estradiol and mestranol in pharmaceutical tablet mixtures. Chloroform solutions of the estrogen were automatically

extracted with alcohol:sulfuric acid (10:90) and analyzed at a rate of twenty samples per hour (Beyer, 1968).

The colorimetric assay of 4,6-unsaturated-3-keto steroids using sodium picrate reagent was initiated by Schulz and Diaz in 1964. This procedure was applied specifically to tablets containing 2.0 mg. of chlormadinone acetate and 80 mg. of mestranol. Neither the estrogen nor tablet excipients of cornstarch, lactose, and magnesium stearate gave a response to the reagent.

The ultraviolet spectrum of the isolated complex showed a maximum at the same wavelength (284 $m\mu$) as that of unreacted chlormadinone, suggesting an intact structure of the 4,6-unsaturated-3-keto moiety in the complexation product.

Although nonphenolic steroids can interfere with the iron-Kober reagent (Venning et al.; 1937), the official USP procedure for the assay of ethinyl estradiol, estradiol benzoate, estradiol dipropionate, and estrone are based on this color reaction (United States Pharmacopeia XVII). Konii (1961) also reported the application of this reagent to ethinyl estradiol. Modifications of the Kober reaction were developed by Longecker (1961) and McKerns (1963).

Rehm and Smith (1960) observed the formation of products with extremely stable colors through the coupling of diazotized 4-amino-6-chloro-m-benzene disulfonamide with phenolic compounds. This reagent was applied to the determination of a number of estrogens by Urbanyi and Rehm in 1966. In general, colorimetric analyses were rapid, accurate, and sensitive.

Various ultraviolet spectrophotometric analyses were also applied to determine the synthetic steroids. Kuhnert-Brandstätter et al.

(1965) reported seventy-seven steroid hormones that are difficult to characterize, especially within a series, because of their strong chemical similarity. They rationalized that the combining of thermomicroscopic and spectrophotometric methods for the identification of these substances would be advantageous. However, the tendency for the high-melting steroids to decompose at their melting temperature often hindered attempts at refractive index determination. Since the materials under investigation contained chromophores, the use of ultraviolet spectrophotometry proved valuable as a substitute or as a correlative to refractive index determinations. Their results for oral contraceptive steroids are listed in Table I.

It was found that the refractive indices of the melt were of more value than ultraviolet absorption data for the differentiation of this class of compounds.

Through UV spectrophotometry, Legrand, Delaroff and Smolik (1958) resolved estradiol-type steroids in the presence of large amounts of ketosteroids, after reduction of the interfering ketone with potassium borohydride. Residual absorption by the reduction products was corrected graphically using data obtained on pure reduced samples of phenolic and ketosteroids.

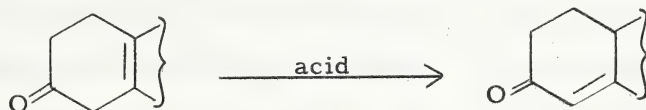
This reduction is applicable to norethynodrel but pure samples of the drug, needed for obtaining data on the background absorption, are normally manufactured containing up to 1.5 per cent of mestranol. Repeated recrystallization will not usually bring this below 0.5 per cent. The method of determining norethynodrel by direct ultraviolet absorption is unsatisfactory due to the lack of characteristic absorption and for this reason Chissell (1964) proposed a colorimetric method using m-dinitro-

TABLE I

Determined Refractive Index and Ultraviolet Values for Oral Contraceptive Steroids

Melting temp. °C	Compound	Eutectic temp. °C with		Glass Powder (n_D)	Temp. °C		UV Absorption Data
		Benzanilide	Salophen		Red Light	Sodium Light	
185	Ethinyl Estradiol	136	151	1.5403	177- 178	179- 180	max. 280 m μ , ϵ = 2,050 (small peak 287 m μ ϵ = 1,800)
202-207	Norethindrone	165	188	1.5101	200- 202	203- 205	max. 240 m μ , ϵ = 16,670

benzene. However, under acid conditions norethynodrel rearranges to norethindrone which has the characteristic conjugated ketone absorption at about $240\text{ m}\mu$. The conjugated ketone absorption is then suitable for the quantitative estimation of norethynodrel.



Bastow (1967) applied this knowledge to the estimation of norethynodrel and mestranol in tablet mixtures. To a powdered mixture of the steroids, hydrochloric acid was added to rearrange the $\Delta^{5,10}$ system to a $\Delta^{4,5}$ -ketone system, with the subsequent determination of norethindrone in methanol at $241\text{ m}\mu$. After eliminating the interfering ketonic absorption of norethynodrel by reduction with borohydride in another sample, mestranol was analyzed. Absorption readings in a methanol solution showed a main peak at $280\text{ m}\mu$, followed by a shoulder at $288\text{ m}\mu$. The excipients of lactose, starch, alginic acid, magnesium stearate and stearic acid did not interfere with the procedure.

A comparative assay on tablets containing mestranol and norethindrone was performed by Shroff and Grodsky (1967), employing ultraviolet spectrophotometric and gas-liquid chromatographic methods. They found that after storage from six weeks at 100°F . to eighteen months at room temperature, analyses by the ultraviolet method indicated reduced recovery of the theoretical amount of mestranol present. From gas-liquid chromatographic results it was evident that mestranol was completely extracted and that it did not undergo decomposition on storage. However, during GLC assays on aged tablets prominent unidentified peaks appeared near the solvent front.

These were later identified as having been generated from a complex formed between polyvinylpyrrolidone and magnesium stearate. The complex had an absorption in the ultraviolet region which distorted the base line and invalidated correction factors. Low results were attributed thereby to base line distortions. A comparison of the two methods suggests that the ultraviolet assay is not reliable for aged tablets because of the interfering chromophore. Nevertheless, it is suitable for fresh tablets.

Keay (1968) reported an analytical evaluation of gestogens in oral contraceptives. His method consisted of solvent extraction of the active ingredients from tablet excipients, followed by identification of the gestogens by thin-layer chromatography or infrared spectrophotometry. The identified steroids were then determined quantitatively by ultraviolet spectrophotometry.

Ultraviolet assay values for progestogens in methanol are shown in Table II.

Values for estrogens are shown in Table III.

A solution of ethynodiol diacetate in methanol showed no peaks in the ultraviolet region.

Spectrofluorometric methods have also been chosen by many investigators, as fluorometry offers a means to the qualitative and quantitative determination of microgram amounts of steroids. Fluorometric methods may be classified according to whether the fluorescence measured is that of the compound in its native state or is induced by chemical transformations. Most accepted procedures for the quantitation of steroids fall into the second category.

TABLE II

Determined Ultraviolet Values for Progestogens

<u>Progestogen</u>	<u>Wavelength of Maximum Extinction, mμ</u>	<u>E^{1%} 1 cm</u>
Norethindrone	240	570
Norethynodrel (isomerised to norethindrone)	240	560
Norethindrone Acetate	240	505
Chlormadinone Acetate	285	510
Megestrol Acetate	289	650

TABLE III

Determined Ultraviolet Values for Estrogens

<u>Estrogen</u>	<u>Solvent</u>	<u>Wavelength of Maximum Extinction, mμ</u>	<u>E₁^{1%} 1 cm</u>
Mestranol (main peak)	methanol	279	82
Mestranol (shoulder peak)	methanol	287.5	14.4
Ethinyl Estradiol (main peak)	methanol	281.5	89
Ethinyl Estradiol (shoulder peak)	methanol	288.5	11.4
Ethinyl Estradiol	0.1N NaOH	298.5	81

Sulfuric acid has been used by many workers (Linford, 1952; Linford and Paulson, 1952; Slaunwhite et al., 1953; Sweat, 1954) to form the fluorogen used for the determination of various steroids. Touchstone and Murawec (1960) found that certain steroids exhibited a greater fluorescent intensity when heated with 2N methanolic potassium hydroxide solution prior to being dissolved in sulfuric acid than when dissolved in sulfuric acid alone. The use of hydrochloric acid to induce fluorescence in steroids has also been reported (Jenson, 1952; Jenson, 1953; Wells et al., 1961; Tishler et al., 1962).

Since all of the anti-fertility products contain progestational steroid hormones whose concentration is 50 to 500 times greater than that of ethinyl estradiol or mestranol, the analysis of the estrogen in this environment presents a difficult analytical problem. Khoury and Cali (1967) developed an automated procedure for the assessment of either estrogen. The method is based on the chemically induced fluorescence exhibited by the phenolsteroid in 90 per cent sulfuric acid.

Mestranol was determined fluorometrically by Hüttenrauch and Keiner (1965) by employing antimony trichloride in glacial acetic acid as the fluorogenic reagent. Comer et al. (1968) has also reported an automated fluorometric method for mestranol in which the fluorescence was developed by a sulfuric acid:methanol (70:30) reagent. Templeton et al. (1968) compared the use of methanol-sulfuric acid (50:50) reagent for the assay of mestranol in tablets by fluorometry, gas chromatography, and colorimetry.

Since the progestogen, d-norgestrel (as dl-racemate), is found in microgram quantities in contraceptive tablets, a fluorometric method has been developed to assay quantitatively Δ^4 -3-ketosteroids

which have both a 17β -hydroxyl and 17α -alkyl or alkyne substitution and $\Delta^{1,3,5(10)}$ -triene-3-ol steroids (Cullen et al., 1968).

The characteristic Δ^4 -3-keto group found in steroids structurally related to d-norgestrel has been ascertained by the 2,4-dinitrophenylhydrazine (Gornall and MacDonald, 1953) and isonicotinic acid hydrazide (Umberger, 1955) colorimetric methods. Unfortunately, these procedures lack the necessary sensitivity for single-tablet analyses of d-norgestrel at the commercial dosage level. A direct ultraviolet spectrophotometric procedure as well as the ultraviolet measurement of the salicyloyl hydrazone (Chen, 1959) or thiosemicarbazone (Bush, 1953) derivatives of the α,β -unsaturated carbonyl group did not exhibit the sensitivity or specificity required in the presence of tablet excipients for an accurate analytical method.

Bush and Sandberg (1953) noted that paper chromatograms sprayed with sodium hydroxide developed an orange-yellow fluorescence which was specific for Δ^4 -3-ketosteroids when examined under UV irradiation. Subsequently, Abelson and Bondy (1955) found that potassium tert-butoxide could produce an alkaline fluorescence reaction with the same steroids in a satisfactory manner. However, the procedure requires meticulous care for satisfactory results, and it is insufficiently rapid and precise for the analysis of single tablets. Smith and Foell (1959) demonstrated that Δ^4 -3-ketones and $\Delta^{1,4}$ -3-ketones exhibit a fluorescence under UV light on paper chromatograms sprayed with isonicotinic acid hydrazide in acid alcohol.

A study of the sulfuric acid-induced fluorescence procedure used in the USP as an identification reaction for hydrocortisone led to the development of a sulfuric acid-ethanol-methanol fluorogenic

reagent suitable for quantitative measurement of microgram amounts of d-norgestrel in tablets of low dosage. Fluorescent properties of structurally related steroids were studied to determine the selectivity of this reaction. It was found that, although d-norgestrel gave a relative fluorescent intensity of 100, many other steroids also gave readings, including norethindrone (58), ethinyl estradiol (660) and mestranol (880). Therefore, this method would not be applicable to d-norgestrel-estrogen mixtures unless complete separation of steroids was achieved prior to the fluorometric measurements.

The infrared spectra of complex molecules examined in the solid state are known to be subject to the effects of polymorphism. Amongst the steroids, spectral evidence of polymorphism has been reported for cortisone acetate (Garratt and Marshall, 1954; Callow and Kennard, 1961), estradiol (Smakula et al., 1957) ethinyl estradiol (Pheasant, 1950; Röpke and Neudert, 1959), and methylprednisolone (Higuchi et al., 1963). In some instances, changes in the spectrum were ascribed to conversion of a crystalline form into an amorphous form or into a second crystalline form (Baker, 1957). Changes in crystalline form and thus in absorption spectrum might also be induced by the solvent extraction methods used for the isolation of steroids from pharmaceutical preparations before infrared examination.

It has been recommended that the effects of polymorphism on the infrared spectra of steroids should be avoided by the use of solutions rather than solid state samples (Page, 1957) or by prolonged grinding of the solid if a mull or potassium bromide disc must be used (Roberts, 1957; Baker, 1957).

After examining related steroids in solution, mull, and disc, and finding the majority of these compounds to exhibit polymorphism (Mesley and Johnson, 1965), Mesley (1966) recorded the solid state infrared spectra and investigated the various polymorphic forms of thirty-five steroids. He found that the grinding involved in the preparation of potassium bromide discs caused spectral changes in many of the compounds. Of the steroids studied, dimethisterone was found to have two polymorphic forms, norethindrone, one form, norethynodrel, one form, and ethinyl estradiol, three forms.

Crystallographically, different solid forms of a particular compound are characterized by differences in the dimensions of the unit lattice cell. In chemical terms, such differences are likely to be due to differing types of association between the individual molecules comprising the unit cell, and these in turn should be reflected in the infrared spectra.

The most common form of association in the majority of the compounds is probably intermolecular hydrogen bonding between hydroxyl groups. This may take the form of either dimeric or polymeric association. With poly-hydroxy compounds there is also the possibility of intramolecular hydrogen bonding. Hydrogen bonding between a hydroxyl group and a carbonyl group is possible in many steroids, particularly between 17α -hydroxyl and 20-keto groups. Bonding of hydrogen to atoms other than oxygen is apparently limited to those compounds which contain fluorine.

It is evident, therefore, that in steroids where different types of association are possible, polymorphism is the rule rather than the exception.

Various chromatographic methods have also been used to separate and analyze steroids. Roberts and Florey (1962) quantitatively estimated hormone preparations using paper chromatographic separation techniques followed by spectrophotometric analysis.

The development of the spectrophotofluorometer, capable of activating and measuring fluorescence throughout the visible and ultraviolet regions, has revealed the presence of useful ultraviolet fluorescence in many compounds not previously known to fluoresce in solution. Duggan et al. (1957), in studying the fluorescent properties of a number of compounds of biological interest, found that estradiol fluoresced in the ultraviolet region in the absence of sulfuric acid. Based on this observation, Roberts and Siino (1963) developed a sensitive quantitative paper chromatographic assay for the routine analyses of estradiol valerate in castor oil formulations and ethinyl estradiol in tablets. The procedure involved four steps: (a) the separation of the intact estrogenic hormone from the interfering degradation products through solvent extraction with N,N-dimethylformamide, (b) the location of the intact estrogenic hormone on the chromatogram by the guide strip technique employing the chromogenic agent diluted phenol-ammonia-concentrated ammonium hydroxide, (c) the elution of the estrogenic hormone from the chromatogram, and (d) the quantitative spectrophotofluorometric analysis of the eluate.

The first quantitative use of the thin layer chromatographic technique was reported by Kirchner et al. in 1954, when he described the assay of biphenyl in citrus fruits and fruit products. Since then, only a minimum number of reports have been published on the quantitative use of TLC for steroidal compounds, including that of Matthews

and associates in 1962.

Bird et al. (1963) applied quantitative thin-layer chromatography to the assay of chlormadinone acetate, alone, and in mixtures with mestranol. Chloroform steroid solutions were spotted on Silica Gel G, developed in a mixture of 9:1 chloroform: anhydrous ethyl ether, dried, and examined rapidly under short wavelength ultraviolet light. The adsorbent containing the spot was removed, the steroid was eluted with absolute ethanol, and absorbances were measured at 283 m μ on the spectrophotometer.

Thin-layer chromatography of steroidal pharmaceuticals not employed in oral contraception was reported by Hara and Mibe in 1967 and 1968. The precision, accuracy, and linearity studies showed that quantitative TLC could be applied to practical quality control.

Of all the preceding methods used to analyze steroid mixtures, gas-liquid chromatography is considered to be the most desirable technique, since it is rapid and it offers the advantage of quantitation obtained concurrently with fractionation.

Since VandenHeuvel et al. (1960) first described a practical separation of steroids by GLC, the technique has been widely applied. Estrogens have been separated without modification (VandenHeuvel et al., 1960), but steroids containing two or more strongly polar functional groups, such as hydroxyl or carboxyl, usually have impractically long retention times and are apt to deteriorate during the chromatographic procedure. To avoid these difficulties the carboxyl groups can be esterified prior to analysis (Sjövall et al., 1961). Hydroxyl groups can be substituted in several ways. Sometimes a simple acetylation is sufficient (Wotiz and Martin 1961).

Derivatives with a considerably higher volatility are obtained when the acylation is performed with trifluoroacetic anhydride (VandenHeuvel et al., 1961). In another technique, hydroxyl groups are converted to methylether groups (Clayton, 1962). The most widely employed method to increase the chromatographic mobility is to substitute the active hydrogen by trimethylsilylether groups (TMS groups). As described by Luukkainen et al. (1961), TMS-derivatives of steroids can be prepared by treatment with a mixture of hexamethyldisilazane and trimethylchlorosilane in a suitable solvent. A newer method introduced by Klebe and coworkers (1966) uses only bis (trimethylsilyl) acetamide. The latter reagent seems to be far more reactive and does not yield acid by-products, such as hydrochloric acid, which can promote undesired side reactions.

Keto groups can be characterized by the formation of N,N-dimethylhydrazones (VandenHeuvel and Horning, 1963) or O-methyl oximes (Fales and Luukkainen, 1965).

Schulz (1965) reported a gas chromatographic method, using 1 per cent QF-1 as the liquid phase, for the rapid quantitation of mestranol in the presence of norethindrone or chlormadinone acetate.

Talmage, Penner, and Geller (1965) used 4 per cent SE-30 in the GLC resolution of ethinyl estradiol in both sesame oil and solid dosage forms. The method involved preliminary separation of the active principle from the oily vehicle or table excipients, addition of an internal standard, acetylation, and final chromatography. Quantitation was accomplished by integration of the areas under the peaks. The accuracy and precision of the method are reported to be superior to the iron-phenol procedure described in the USP.

Ethinyl estradiol, extracted from other tablet excipients and quantitatively determined by the gas chromatography of its trimethylsilyl ether on 3.8 per cent SE-30, was reported by Boughton et al. (1966). A comparison of this analysis with the USP method indicated that, in general, the precision of the USP procedure was superior to that of the gas chromatographic method.

The retention times of C₁₈ and C₁₉ series of steroids were measured systematically by gas chromatography, and the correlation between retention time and the molecular structure was examined (Hara et al., 1967). Steroid compounds were first analyzed unchanged and then with converted functional groups, on both 1.0 per cent SE-30 and 1.5 per cent QF-1. In general, the retention values obtained for a given functional group were approximately the same.

Other analyses of the oral contraceptive steroids include an ion-exchange method for estrogens (Sjöstrom and Nykanen, 1957), the nuclear magnetic resonance of several steroids in various solvents (Cross et al., 1965), various polarographic procedures for Δ^4 -3-keto-steroids (Eisenbrand and Picher, 1939; Kolthoff and Lingane, 1952), and a report on the physical properties of d-norgestrel, including melting point, thin-layer chromatography, proton magnetic resonance, infrared, ultraviolet and fluorescence spectroscopy (Cullen et al., 1968).

The official British Pharmacopoeia (1968) assays of ethinyl estradiol, mestranol, norethindrone, norethindrone acetate and norethynodrel include the dissolution of an accurately weighed amount of steroid in tetrahydrofuran, addition of silver nitrate solution, and potentiometric titration with N/10 sodium hydroxide. In contrast, the

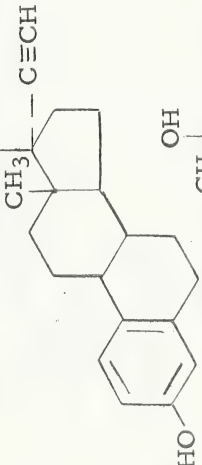
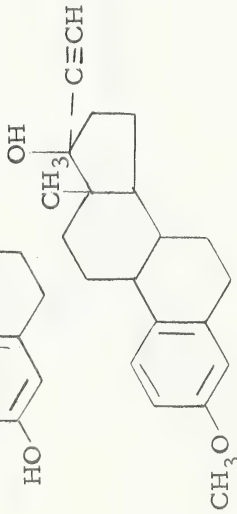
BP assay for dimethisterone utilizes an ultraviolet spectrophotometric measurement of the steroid at 240 m μ .

3. STRUCTURAL FORMULAS

Estrogenic and progestational steroids currently available on the Canadian market for contraceptive purposes are listed in Tables IV and V.

TABLE IV

Synthetic Estrogens Currently Available as Oral Contraceptives in Canada

<u>Generic Name</u>	<u>Structural Formula</u>	<u>*Chemical Name</u>
1. Ethinyl Estradiol (Ethinyl Estradiol)		19-Nor-17 α -pregna-1,3,5(10)-trien-20-yne-3,17-diol
2. Mestranol		3-Methoxy-19-nor-17 α -pregna-1,3,5(10)-trien-20-yne-17-ol

* Many of these steroid chemical names are patterned after pregnane.

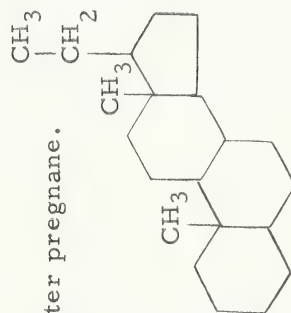
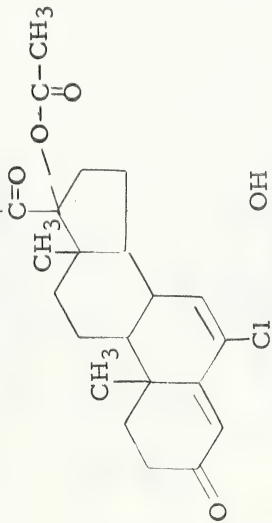
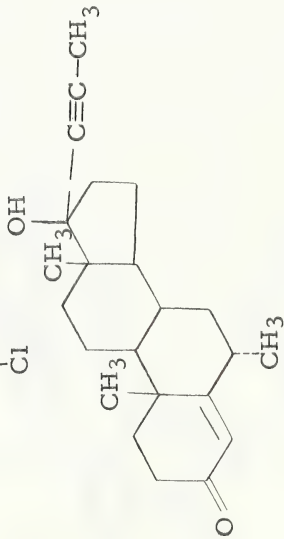
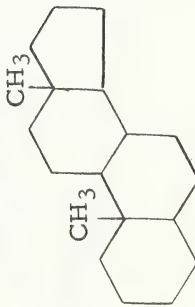


TABLE V

Synthetic Progestogens (Progestins) Currently Available as Oral Contraceptives in Canada

<u>Generic Name</u>	<u>Structural Formula</u> CH_3	<u>Chemical Name</u>
1. Chlormadinone Acetate		6-Chloro-17 α -hydroxy acetate-pregna-4,6- diene-3,20-dione
2. Dimethisterone		*17 β -Hydroxy-6 α - methyl-17-(1-propynyl)- androst-4-en-3-one
		

* patterned after androstane

TABLE V - Continued

<u>Generic Name</u>	<u>Structural Formula</u>	<u>Chemical Name</u>
3. Ethynodiol Diacetate		19-Nor-17 α -pregn-4-en-20-yne-3 β ,17-diol diacetate
4. Megestrol Acetate		6-Methyl-17 α -hydroxy-acetate-pregna-4,6-diene-3,20-dione
5. Norethindrone		17 β -Hydroxy-19-nor-17 α -pregna-4-en-20-yn-3-one

TABLE V - Continued

Generic Name	Structural Formula	Chemical Name
6. Norethindrone Acetate		17 β -Hydroxyacetate- 19-nor-17 α -pregna-4- en-20-yn-3-one
7. Norethynodrel (Norethindrel)		17 β -Hydroxy-19-nor- 17 α -pregna-5(10)-en- 20-yn-3-one
8. d-Norgestrel (as <u>dl</u> -racemate)		13-Ethyl-17-hydroxy- 18,19-dinor-17 α - pregna-4-en-20-yn- 3-one

STATEMENT OF THE PROBLEM

The aims and objectives of this investigation were twofold:

- a) to develop a quantitative analytical method for the identification of the oral contraceptive steroids ,
- and, b) to apply this method to commercially-available anti-fertility preparations .

For many years , infrared spectrophotometry has been a well-known and widely-used technique in the identification of organic compounds . It has recently been introduced into the United States Pharmacopeia and the British Pharmacopoeia as an official qualitative test and a quantitative assay for many substances .

In this work , an infrared spectrophotometric assay was developed in an attempt to quantitatively estimate single steroids and steroid mixtures . However , it became evident that even though an estrogen could be measured by infrared irradiation when found singly in an oral contraceptive preparation , the assay required the use of large numbers of tablets . A more sensitive analytical method , necessary as a correlative to the infrared analysis , was chosen in gas-liquid chromatography .

As evidenced by an imposing number of publications , GLC has recently found wide application in the analysis of steroidal compounds . , Quantitative analysis of steroid hormones in biological fluids has been achieved by this method , showing GLC to be an efficient substitute for the painstaking and time-consuming work required in separation procedures . Of the four gas-liquid chromatographic publications involving oral contraceptives , all were concerned with analyzing only the estrogen component . In this investigation , efforts were made to develop a gas chromatographic procedure which would measure quantitatively both estrogen and progestogen as they are found in pharmaceutical dosage forms .

EXPERIMENTAL

I. INFRARED SPECTROPHOTOMETRY

Apparatus

Beckman IR-10 Infrared Spectrophotometer; Beckman 0.5 mm. matched infrared solution cells; polystyrene calibration film; vacuum tubing for drying cells; Carver Hydraulic Laboratory Press; Beckman Die Apparatus; Wig-L-Bug Amalgamator; Adams Dynac Centrifuge; Gram-atic Balance; Swinny Filter Holder with a 13 mm. diameter Millipore filter membrane, Type GS, 0.22 μ pore size; magnetic stirring apparatus; mortar and pestle; siliconized laboratory glass-ware including beakers, pipettes, 1 ml., 2ml., 5 ml., and 10 ml. volumetric flasks, Pasteur pipettes and 15 ml. centrifuge tubes.

Chemicals

Potassium Bromide Powder for Infrared Spectroscopy (Matheson Coleman and Bell).

Reagents

Certified A.C.S. Spectroanalyzed Chloroform (Fisher Scientific Company).

Reference Standards

The following is a list of the pure drugs employed as reference standards.

Pure Drug

Manufacturer

I. Estrogens

Ethinyl Estradiol USP.

Delmar Chemicals

Mestranol

Searle

Continued . . .

Pure DrugManufacturerII. Progestogens

Chlormadinone Acetate	Lilly
Dimethisterone	Mead Johnson
Ethinodiol Diacetate	Searle
Megestrol Acetate	Mead Johnson
Norethindrone	Ortho
Norethindrone Acetate	Syntex
Norethynodrel	Searle
d-Norgestrel (as <u>dl</u> -racemate)	Wyeth

ProceduresA. Siliconization of Glassware

All glassware was carefully washed in soap and water , rinsed with acetone , and allowed to air dry. Tongs were used in the handling of equipment thus removing the possibility of greasy finger-marks being left on the glassware. When thoroughly dry , the glassware was dipped into a 2 per cent solution of Dow-Corning Silicone Oil 200 in carbon tetrachloride and inverted to drip dry for twenty-four hours. The silicone coating was baked on by heating in an oven at 500^oC. for four hours.

One silicone treatment was sufficient for the length of the research project.

B. Infrared Spectra of Probable Tablet Excipients

Potassium bromide discs of suitable concentration , varying from 0.25 per cent to 2.00 per cent , were prepared for probable tablet excipients. Included in the analysis were sucrose , dibasic calcium phosphate ,

mannitol, stearic acid, talc, lactose, kaolin, corn starch, and calcium and magnesium stearate.

Approximately 1 mg. of excipient was accurately weighed, 200 to 800 mg. of potassium bromide was added, and the components were thoroughly mixed in a Wig-L-Bug Amalgamator for two minutes. The powder was subsequently placed in a press and die apparatus for fifteen minutes at a pressure of 90,000 psi. After careful removal of the disc from the mold and placement in the disc holder, an infrared spectrum was taken.

This procedure was repeated for each of the likely tablet glidants, lubricants, and fillers.

C. Determination of an Absorption Peak Most Suitable for Quantitative Analysis.

An accurate quantity of pure steroid was weighed into a 10 ml. siliconized volumetric flask. Spectroanalyzed chloroform was added and the mixture was stirred on the magnetic apparatus until the solid was completely dissolved. Additional chloroform was added until a 10 ml. volume of solution was achieved. The stock solution was well mixed prior to making appropriate dilutions with chloroform to bring the concentration of the steroid to approximately 1.0 mg./ml. solution. The spectra of all solutions were taken on the infrared spectrophotometer against a reference blank of chloroform. All spectra were calibrated with a polystyrene template to correct for wavenumber accuracy. Between readings, the infrared cell was emptied and thoroughly vacuum dried.

In each instance, the strongest absorption peak which would be least likely interfered with by the tablet excipients was chosen for

quantitative analysis. A calibration curve was prepared with absorbance being read at the selected peak. If the plot of absorbance versus concentration of steroid produced a straight-line relationship for a reasonable concentration range, the absorbance peak was judged suitable for quantitative analysis.

D. Preparation of Calibration Curves

All calibration curves were prepared in triplicate, and a composite calibration curve prepared from the three results.

1. Estrogens

(i) Calibration Curve for Ethinyl Estradiol

Exactly 0.200 g. of ethinyl estradiol powder was weighed into a 10 ml. volumetric flask. Approximately 8 ml. of chloroform was added to the flask, and the mixture was stirred for fifteen minutes until all the solid dissolved. The solution was brought to volume with chloroform and the flask was shaken. 4.0 ml. of the stock solution (solution A) was pipetted into a 5 ml. volumetric flask; 5.0 ml. of the stock solution was pipetted into a 10 ml. volumetric flask. The contents of the flasks were brought to volume with chloroform and then manually shaken, producing solution B and solution C, respectively. Into two separate 10 ml. volumetric flasks, aliquots of 5.0 ml. and 3.0 ml. of solution C were added. Each flask was brought to volume with chloroform producing solution D and solution E, respectively.

Stock Solution and Dilutions for Ethinyl Estradiol

<u>Solution</u>		<u>Concentration</u>
A	0.200 g. qs. to 10 ml.	20.0 mg./ml.
B	4.0 ml. (A) qs. to 5 ml.	16.0 mg./ml.
C	5.0 ml. (A) qs. to 10 ml.	10.0 mg./ml.
D	5.0 ml. (C) qs. to 10 ml.	5.0 mg./ml.
E	3.0 ml. (C) qs. to 10 ml.	3.0 mg./ml.

Immediately following preparation, the contents of the flasks were transferred, in turn, to a 0.5 mm. Beckman infrared cell. Two spectra of each solution were taken against a 0.5 mm. reference cell filled with chloroform. Between readings, the sample cell was emptied and thoroughly vacuum dried.

Spectra were obtained for the region between 1800 cm^{-1} and 1500 cm^{-1} .

(ii) Calibration Curve for Mestranol

The procedure employed in the preparation of a calibration curve for mestranol was identical to that used in Part (i), Infrared Spectrophotometry, with the exception of the concentration of the stock solution and subsequent dilutions. All aliquots were made to volume with chloroform before continuing dilution.

Stock Solution and Dilutions for Mestranol

<u>Solution</u>		<u>Concentration</u>
A	0.500 g. qs. to 10 ml.	50.0 mg./ml.
B	6.0 ml. (A) qs. to 10 ml.	30.0 mg./ml.
C	2.0 ml. (A) + 3.0 ml. (B) qs. to 10 ml.	19.0 mg./ml.
D	5.0 ml (B) qs. to 10 ml.	15.0 mg./ml.

Continued . . .

Stock Solution and Dilutions for Mestranol - Continued

<u>Solution</u>		<u>Concentration</u>
E	1.0 ml. (A) + 4.0 ml. (D) qs. to 10 ml.	11.0 mg./ml.
F	4.0 ml. (D) qs. to 10 ml.	6.0 mg./ml.
G	5.0 ml. (F) qs. to 10 ml.	3.0 mg./ml.
H	5.0 ml. (G) qs. to 10 ml.	1.5 mg./ml.

Infrared spectra of solutions C to H were taken between 1800 cm^{-1} and 1450 cm^{-1} .

2. Progestogens(iii) Calibration Curve for Chlormadinone Acetate

The procedure employed in the preparation of a calibration curve for chlormadinone acetate was identical to that used in Part (i), Infrared Spectrophotometry, with the exception of concentration of the stock solution and subsequent dilutions. All aliquots were made to volume with chloroform before continuing dilution.

Stock Solution and Dilutions for Chlormadinone Acetate

<u>Solution</u>		<u>Concentration</u>
A	0.200 g. qs. to 10 ml.	20.0 mg./ml.
B	5.0 ml. (A) qs. to 10 ml.	10.0 mg./ml.
C	4.0 ml. (A) qs. to 10 ml.	8.0 mg./ml.
D	5.0 ml. (B) qs. to 10 ml.	5.0 mg./ml.
E	5.0 ml. (D) qs. to 10 ml.	2.5 mg./ml.
F	5.0 ml. (E) qs. to 10 ml.	1.25 mg./ml. rounded off to 1.3 mg./ml.

Infrared spectra of solutions B to F were taken between 1900 cm^{-1} and 1450 cm^{-1} .

(iv) Calibration Curve for Dimethisterone

The procedure employed in the preparation of a calibration curve for dimethisterone was identical to that used in Part (i), Infrared Spectrophotometry, with the exception of concentration of the stock solution and subsequent dilutions. All aliquots were made to volume with chloroform before continuing dilution.

Stock Solution and Dilutions for Dimethisterone

<u>Solution</u>		<u>Concentration</u>
A	0.250 g. qs. to 10 ml.	25.0 mg./ml.
B	6.0 ml. (A) qs. to 10 ml.	15.0 mg./ml.
C	8.0 ml. (B) qs. to 10 ml.	12.0 mg./ml.
D	8.0 ml. (C) qs. to 10 ml.	9.6 mg./ml.
E	3.0 ml. (A) qs. to 10 ml.	7.5 mg./ml.
F	8.0 ml. (E) qs. to 10 ml.	6.0 mg./ml.
G	5.0 ml. (D) qs. to 10 ml.	4.8 mg./ml.
H	7.0 ml. (F) qs. to 10 ml.	4.2 mg./ml.
I	2.0 ml. (D) + 4.0 ml. (H) qs. to 10 ml.	3.6 mg./ml.
J	3.0 ml. (G) + 3.0 ml. (H) qs. to 10 ml.	2.7 mg./ml.
K	6.0 ml. (J) + 1.0 ml. (G) qs. to 10 ml.	2.1 mg./ml.
L	5.0 ml. (K) qs. to 10 ml.	1.05 mg./ml. rounded off to 1.1 mg./ml.

Infrared spectra of solutions G to L were taken between 1850 cm^{-1} and 1450 cm^{-1} .

(v) Calibration Curve for Ethynodiol Diacetate

The procedure employed in the preparation of a calibration curve for ethynodiol diacetate was identical to that used in Part (i),

Infrared Spectrophotometry, with the exception of concentration of the stock solution and subsequent dilutions. All aliquots were made to volume with chloroform before continuing dilution.

Stock Solution and Dilutions for Ethynodiol Diacetate

<u>Solution</u>		<u>Concentration</u>
A	0.500 g. qs. to 10 ml.	50.0 mg./ml.
B	4.0 ml. (A) qs. to 10 ml.	20.0 mg./ml.
C	6.0 ml. (B) qs. to 10 ml.	12.0 mg./ml.
D	5.0 ml. (C) qs. to 10 ml.	6.0 mg./ml.
E	1.0 ml. (A) qs. to 10 ml.	5.0 mg./ml.
F	2.0 ml. (B) qs. to 10 ml.	4.0 mg./ml.
G	5.0 ml. (D) qs. to 10 ml.	3.0 mg./ml.
H	5.0 ml. (E) qs. to 10 ml.	2.5 mg./ml.
I	5.0 ml. (F) qs. to 10 ml.	2.0 mg./ml.
J	5.0 ml. (G) qs. to 10 ml.	1.5 mg./ml.
K	5.0 ml. (I) qs. to 10 ml.	1.0 mg./ml.
L	5.0 ml. (K) qs. to 10 ml.	0.5 mg./ml.
M	5.0 ml. (L) qs. to 10 ml.	0.25 mg./ml. rounded off to 0.3 mg./ml.

Infrared spectra of solutions D to M were taken between 1900 cm^{-1} and 1500 cm^{-1} .

(vi) Calibration Curve for Megestrol Acetate

The procedure employed in the preparation of a calibration curve for megestrol acetate was identical to that used in Part (i), Infrared Spectrophotometry, with the exception of concentration of the stock solution and subsequent dilutions. All aliquots were made to volume with chloroform before continuing dilution.

Stock Solution and Dilutions for Megestrol Acetate

<u>Solution</u>		<u>Concentration</u>
A	0.100 g. qs. to 10 ml.	10.0 mg./ml.
B	5.0 ml. (A) qs. to 10 ml.	5.0 mg./ml.
C	4.0 ml. (A) qs. to 10 ml.	4.0 mg./ml.
D	6.0 ml. (B) qs. to 10 ml.	3.0 mg./ml.
E	5.0 ml. (C) qs. to 10 ml.	2.0 mg./ml.
F	5.0 ml. (E) qs. to 10 ml.	1.0 mg./ml.

Infrared spectra of solutions B to F were taken between 1850 cm^{-1} and 1550 cm^{-1} .

(vii) Calibration Curve for Norethindrone

The procedure employed in the preparation of a calibration curve for norethindrone was identical to that used in Part (i), Infrared Spectrophotometry, with the exception of concentration of the stock solution and subsequent dilutions. All aliquots were made to volume with chloroform before continuing dilution.

Stock Solution and Dilutions for Norethindrone

<u>Solution</u>		<u>Concentration</u>
A	0.500 g. qs. to 10 ml.	50.0 mg./ml.
B	2.0 ml. (A) qs. to 10 ml.	10.0 mg./ml.
C	7.0 ml. (B) qs. to 10 ml.	7.0 mg./ml.
D	1.0 ml. (A) qs. to 10 ml.	5.0 mg./ml.
E	5.0 ml. (C) qs. to 10 ml.	3.5 mg./ml.
F	5.0 ml. (D) qs. to 10 ml.	2.5 mg./ml.
G	2.0 ml. (D) qs. to 10 ml.	1.0 mg./ml.
H	5.0 ml. (G) qs. to 10 ml.	0.5 mg./ml.

Infrared spectra of solutions D to H were taken between 1850 cm^{-1} and 1450 cm^{-1} .

(viii) Calibration Curve for Norethindrone Acetate

The procedure employed in the preparation of a calibration curve for norethindrone acetate was identical to that used in Part (i) , Infrared Spectrophotometry , with the exception of concentration of the stock solution and subsequent dilutions. All aliquots were made to volume with chloroform before continuing dilution.

Stock Solution and Dilutions for Norethindrone Acetate

<u>Solution</u>		<u>Concentration</u>
A	0.200 g. qs. to 10 ml.	20.0 mg./ml.
B	5.0 ml. (A) qs. to 10 ml.	10.0 mg./ml.
C	4.0 ml. (A) qs. to 10 ml.	8.0 mg./ml.
D	8.0 ml. (C) qs. to 10 ml.	6.4 mg./ml.
E	5.0 ml. (B) qs. to 10 ml.	5.0 mg./ml.
F	5.0 ml. (E) qs. to 10 ml.	2.5 mg./ml.
G	5.0 ml. (F) qs. to 10 ml.	1.25 mg./ml. rounded off to 1.3 mg./ml.

Infrared spectra of solutions C to G were taken between 1900 cm^{-1} and 1450 cm^{-1} .

(ix) Calibration Curve for Norethynodrel

The procedure employed in the preparation of a calibration curve for norethynodrel was identical to that used in Part (i) , Infrared Spectrophotometry , with the exception of concentration of the stock solution and subsequent dilutions. All aliquots were made to volume with chloroform before continuing dilution.

Stock Solution and Dilutions for Norethynodrel

<u>Solution</u>		<u>Concentration</u>
A	0.500 g. qs. to 10 ml.	50.0 mg./ml.
B	2.0 ml. (A) qs. to 10 ml.	10.0 mg./ml.
C	7.0 ml. (B) qs. to 10 ml.	7.0 mg./ml.
D	1.0 ml. (A) qs. to 10 ml.	5.0 mg./ml.
E	5.0 ml. (C) qs. to 10 ml.	3.5 mg./ml.
F	5.0 ml. (D) qs. to 10 ml.	2.5 mg./ml.
G	2.0 ml. (D) qs. to 10 ml.	1.0 mg./ml.

Infrared spectra of solutions C to G were taken between 1900 cm^{-1} and 1500 cm^{-1} .

(x) Calibration Curve for d-Norgestrel (as dl-racemate)

The procedure employed in the preparation of a calibration curve for d-norgestrel was identical to that used in Part (i), Infrared Spectrophotometry, with the exception of concentration of the stock solution and subsequent dilutions. All aliquots were made to volume with chloroform before continuing dilution.

Stock Solution and Dilutions for d-Norgestrel

<u>Solution</u>		<u>Concentration</u>
A	0.100 g. qs. to 10 ml.	10.0 mg./ml.
B	5.0 ml. (A) qs. to 10 ml.	5.0 mg./ml.
C	4.0 ml. (A) qs. to 10 ml.	4.0 mg./ml.
D	6.0 ml. (B) qs. to 10 ml.	3.0 mg./ml.
E	5.0 ml. (C) qs. to 10 ml.	2.0 mg./ml.
F	5.0 ml. (E) qs. to 10 ml.	1.0 mg./ml.

Infrared spectra of solutions B to F were taken between 1850 cm^{-1} and 1450 cm^{-1} .

E. Quantitative Assay of Pharmaceutical Preparations

General Procedure 1

An estimated number of oral contraceptive tablets required for a quintuplet assay were accurately weighed. After crushing the tablets with a pestle and mortar, an exact quantity of powder equal to a certain amount of steroid was added to a 10 ml. siliconized volumetric flask. The concentration of steroid was calculated to fall upon the prepared calibration curve.

About one-half of the final required volume of chloroform was added, and the mixture was stirred magnetically for one hour. After complete dissolution of steroids in the solution, the entire mixture was transferred to a 15 ml. siliconized centrifuge tube with adequate rinsing of the initial flask with chloroform. The tube was placed in the centrifuge and rotated at a speed of 1200 rpm for eight minutes. Using a siliconized Pasteur pipette, the upper solution was carefully removed and put into a volumetric flask of appropriate volume. The remaining volume of chloroform necessary to make up the volume of the volumetric flask was divided into two portions. Centrifugation was repeated twice with the two portions of chloroform, and the quantities of chloroform removed from the centrifuge tube were mixed together. The solution of steroids was then made up to volume with chloroform and shaken manually. An immediate infrared analysis of the prospective area containing the chosen quantitative peak followed. Between readings, including two spectra of each solution, the infrared cell was emptied and thoroughly vacuum dried.

The entire assay was repeated five times for each oral contraceptive. This procedure was applied to the trade preparations of

Enovid [®] 5 mg., 10 mg., Enovid-E [®] , Norinyl [®] 1 mg., 2 mg., Norlestrin [®] 1.0 mg., 2.5 mg., Ovulen [®] 0.5 mg., Ovulen [®] Three Week, Norquen [®] , Oracon [®] , Ovex [®] , and Secrovin [®] .

General Procedure 2

The second method of analysis was identical to that in Procedure 1, Infrared Spectrophotometry, except that after centrifugation for eight minutes, the mixture was still not sufficiently separated. Rotation at a speed of 1400 rpm for a total of thirty minutes did not improve the situation. Therefore, after one-half hour, the opaque solution was transferred to a Swinny filter holder fitted with a 13 mm. diameter Millipore filter membrane of 0.22 μ pore size and filtered into a suitable volumetric flask. Centrifugation was repeated twice and each time the opaque solution was filtered. The resultant clear solutions were mixed together and made up to volume with chloroform. Immediate infrared analysis followed.

The entire assay was repeated five times for each oral contraceptive. This procedure was applied to the trade preparations of C-Quens [®] , Ortho-Novum [®] 1 mg., 2 mg., 5 mg., SQ, Ovral [®] , and Serial 28 [®] .

TABLE VI

Trade Preparations Assayed

A. Combination Tablets

<u>Trade Name</u>	<u>Manufacturer</u>	<u>Component Steroids</u>
1. Enovid [®] 5 mg.	Searle	each tablet: 5 mg. Norethynodrel with 0.075 mg. Mestranol
2. Enovid [®] 10 mg.	Searle	each tablet: 9.85 mg. Norethynodrel with 0.15 mg. Mestranol
3. Enovid-E [®]	Searle	each tablet: 2.5 mg. Norethynodrel with 0.1 mg. Mestranol
4. Norinyl [®] 1 mg.	Syntex	each tablet: 1.0 mg. Norethindrone with 0.05 mg. Mestranol
5. Norinyl [®] 2 mg.	Syntex	each tablet: 2.0 mg. Norethindrone with 0.10 mg. Mestranol
6. Norlestrin [®] 1.0 mg.	Park Davis	each tablet: 1.0 mg. Norethindrone Acetate with 0.05 mg. Ethinyl Estradiol
7. Norlestrin [®] 2.5 mg.	Park Davis	each tablet: 2.5 mg. Norethindrone Acetate with 0.05 mg. Ethinyl Estradiol
8. Ortho-Novum [®] 1 mg.	Ortho	each tablet: 1 mg. Norethindrone with 0.05 mg Mestranol

Continued . . .

TABLE VI - Continued

A. Combination Tablets

<u>Trade Name</u>	<u>Manufacturer</u>	<u>Component Steroids</u>
9. Ortho-Novum ® 2 mg.	Ortho	each tablet: 2 mg. Norethindrone with 0.10 mg. Mestranol
10. Ortho-Novum ® 5 mg.	Ortho	each tablet: 5 mg. Norethindrone with 0.075 mg. Mestranol
11. Ovral ®	Wyeth	each tablet: 0.25 mg. d-Norgestrel (as dl-racemate) with 0.05 mg. Ethinyl Estradiol
12. Ovulen ® 0.5 mg.	Searle	each tablet: 0.5 mg. Ethynodiol Diacetate with 0.1 mg. Mestranol
13. Ovulen ® Three Week	Searle	each tablet: 1.0 mg. Ethynodiol Diacetate with 0.1 mg. Mestranol

TABLE VII

Trade Preparations Assayed

B. Sequential Tablets

<u>Trade Name</u>	<u>Manufacturer</u>	<u>Component Steroids</u>
1. C-Quens [®]	Lilly	15 white tablets: 0.08 mg. Mestranol 5 peach tablets: 2 mg. Chlormadinone Acetate with 0.08 mg. Mestranol
2. Norquen [®]	Syntex	14 white tablets: 0.08 mg. Mestranol 7 blue tablets: 2 mg. Norethindrone with 0.08 mg. Mestranol
3. Oracon [®]	Mead Johnson	16 white tablets: 0.1 mg. Ethinyl Estradiol 5 pink tablets: 25 mg. Dimethisterone with 0.1 mg. Ethinyl Estradiol
4. Ortho-Novum [®] SQ	Ortho	14 white tablets: 0.08 mg. Mestranol 6 blue tablets: 2 mg. Norethindrone with 0.08 mg. Mestranol
5. Ovex [®]	Mead Johnson	16 white tablets: 0.1 mg. Ethinyl Estradiol 5 peach tablets: 5 mg. Megestrol Acetate with 0.1 mg. Ethinyl Estradiol
6. Secrovin [®]	The British Drug Houses	16 white tablets: 0.1 mg. Ethinyl Estradiol 5 blue tablets: 25 mg. Dimethisterone with 0.1 mg. Ethinyl Estradiol
7. Serial 28 [®]	The British Drug Houses	16 red tablets: 0.1 mg. Ethinyl Estradiol 5 white tablets: 1.0 mg. Megestrol Acetate with 0.1 mg. Ethinyl Estradiol 7 blue tablets: Lactose

II. GAS-LIQUID CHROMATOGRAPHY

Apparatus

Gas chromatograph, Hewlett-Packard Model 700, with dual flame ionization detector; Hamilton 10 μ l. syringe with Chaney Adapter; siliconized standard laboratory glassware; mortar and pestle; magnetic stirring apparatus; Gram-atic Balance; vacuum pump; wooden 1 ml. and 2 ml. volumetric holders; Adams Dynac Centrifuge.

Column

Silanized glass column (6 feet x $\frac{1}{4}$ inch outer diameter) packed with 3 per cent OV-1* on 80/100 mesh Diatoport S.

Reagent

Certified A.C. S. Spectroanalyzed Chloroform (Fisher Scientific Company).

Internal Standard

5 α -Cholestane (Mann Research)

Reference Standards

The following is a list of the pure drugs employed as reference standards.

	<u>Pure Drug</u>	<u>Manufacturer</u>
I	Estrogen	
	Mestranol	Searle
II	Progestogens	
	Chlormadinone Acetate	Lilly
	Ethynodiol Diacetate	Searle
	Norethindrone	Ortho
	Norethynodrel	Searle

* Dimethylsilicone Gum Rubber Liquid Phase

Procedures

A. Siliconization of Glassware

The method of siliconizing glassware was identical to that described in the Infrared Spectrophotometry section.

B. Internal Standard (Cholestane) Solution

Exactly 0.250 g. of cholestane powder was accurately weighed into a 25 ml. siliconized volumetric flask. Chloroform was added to volume and the solution was manually shaken. 5.0 ml. of the stock solution was pipetted into a 10 ml. volumetric flask, made up to volume with chloroform, and vigorously shaken.

Stock Solution and Dilutions for Cholestane

<u>Solution</u>		<u>Concentration</u>
A	0.250 g. qs. to 25 ml.	10.0 $\mu\text{g.}/\mu\text{l.}$
B	5.0 ml. (A) qs. to 10 ml.	5.0 $\mu\text{g.}/\mu\text{l.}$

C. Preparation of Calibration Curves

(i) Calibration Curve for Chlormadinone Acetate and Mestranol

Exactly 0.250 g. chlormadinone acetate and 0.125 g. mestranol were accurately weighed into a 25 ml. volumetric flask. The solution was made to volume with chloroform and vigorously shaken. Subsequent dilutions were made to volume with chloroform before continuing dilution.

Stock Solution and Dilutions for Chlormadinone

Acetate and Mestranol

<u>Solution</u>		<u>Concentration</u>	
		<u>Chlormadinone Acetate</u>	<u>Mestranol</u>
1	0.25 g. Chlormadinone Acetate plus 0.125 g. Mestranol qs. to 25 ml.	10.0 $\mu\text{g.}/\mu\text{l.}$	5.0 $\mu\text{g.}/\mu\text{l.}$
2	8.0 ml. (1) qs. to 10 ml.	8.0 $\mu\text{g.}/\mu\text{l.}$	4.0 $\mu\text{g.}/\mu\text{l.}$
3	6.0 ml. (1) qs. to 10 ml.	6.0 $\mu\text{g.}/\mu\text{l.}$	3.0 $\mu\text{g.}/\mu\text{l.}$
4	4.0 ml. (1) qs. to 10 ml.	4.0 $\mu\text{g.}/\mu\text{l.}$	2.0 $\mu\text{g.}/\mu\text{l.}$
5	5.0 ml. (4) qs. to 10 ml.	2.0 $\mu\text{g.}/\mu\text{l.}$	1.0 $\mu\text{g.}/\mu\text{l.}$
6	5.0 ml. (5) qs. to 10 ml.	1.0 $\mu\text{g.}/\mu\text{l.}$	0.5 $\mu\text{g.}/\mu\text{l.}$

1.0 ml. of Solution 1 was pipetted into a 2 ml. volumetric tube, to which was added 1.0 ml. of Cholestane Solution B. This procedure was continued for Solutions 2 to 6 until each tube contained a mixture of chlormadinone acetate, mestranol, and cholestane. The final concentrations of chlormadinone acetate, mestranol, and cholestane are shown on page 53.

2.0 μl of Solutions 1_B to 6_B were consecutively injected into the gas chromatograph with conditions of: column temperature, 258°; injection port temperature, 280°; detector temperature, 262°; helium carrier gas at a flow of 30 ml./min.; air flow, 25 ml./min.; and hydrogen flow, 15 ml./min.. When all three peaks of a solution had emerged on the chromatogram, the next solution was injected. Attenuation was adjusted accordingly.

Final Concentrations of Chlormadinone Acetate, Mestranol, and Cholestane

Solution	Chlormadinone Acetate and Mestranol		Cholestane	Concentration		
				Chlormadinone Acetate	Mestranol	Cholestane
1 _B	1.0 ml. Solution 1	+	1.0 ml. Solution B	10.0 $\mu\text{g.}/2\mu\text{l.}$	5.0 $\mu\text{g.}/2\mu\text{l.}$	5.0 $\mu\text{g.}/2\mu\text{l.}$
2 _B	1.0 ml. Solution 2	+	1.0 ml. Solution B	8.0 $\mu\text{g.}/2\mu\text{l.}$	4.0 $\mu\text{g.}/2\mu\text{l.}$	5.0 $\mu\text{g.}/2\mu\text{l.}$
3 _B	1.0 ml. Solution 3	+	1.0 ml. Solution B	6.0 $\mu\text{g.}/2\mu\text{l.}$	3.0 $\mu\text{g.}/2\mu\text{l.}$	5.0 $\mu\text{g.}/2\mu\text{l.}$
4 _B	1.0 ml. Solution 4	+	1.0 ml. Solution B	4.0 $\mu\text{g.}/2\mu\text{l.}$	2.0 $\mu\text{g.}/2\mu\text{l.}$	5.0 $\mu\text{g.}/2\mu\text{l.}$
5 _B	1.0 ml. Solution 5	+	1.0 ml. Solution B	2.0 $\mu\text{g.}/2\mu\text{l.}$	1.0 $\mu\text{g.}/2\mu\text{l.}$	5.0 $\mu\text{g.}/2\mu\text{l.}$
6 _B	1.0 ml. Solution 6	+	1.0 ml. Solution B	1.0 $\mu\text{g.}/2\mu\text{l.}$	0.5 $\mu\text{g.}/2\mu\text{l.}$	5.0 $\mu\text{g.}/2\mu\text{l.}$

(ii) Calibration Curve for Ethynodiol Diacetate and Mestranol

The procedure employed in the preparation of a calibration curve for ethynodiol diacetate and mestranol was identical to that used in Part (i), Gas-liquid Chromatography, for chlormadinone acetate and mestranol. All aliquots were made to volume with chloroform before continuing dilution.

Stock Solution and Dilutions for Ethynodiol Diacetate
and Mestranol

<u>Solution</u>		<u>Concentration</u>	
		<u>Ethynodiol Diacetate</u>	<u>Mestranol</u>
1	0.250 g. Ethynodiol Diacetate plus 0.125 g. Mestranol qs. to 25 ml.	10.0 $\mu\text{g.}/\mu\text{l.}$	5.0 $\mu\text{g.}/\mu\text{l.}$
2	8.0 ml. (1) qs. to 10 ml.	8.0 $\mu\text{g.}/\mu\text{l.}$	4.0 $\mu\text{g.}/\mu\text{l.}$
3	6.0 ml. (1) qs. to 10 ml.	6.0 $\mu\text{g.}/\mu\text{l.}$	3.0 $\mu\text{g.}/\mu\text{l.}$
4	4.0 ml. (1) qs. to 10 ml.	4.0 $\mu\text{g.}/\mu\text{l.}$	2.0 $\mu\text{g.}/\mu\text{l.}$
5	5.0 ml. (4) qs. to 10 ml.	2.0 $\mu\text{g.}/\mu\text{l.}$	1.0 $\mu\text{g.}/\mu\text{l.}$
6	5.0 ml. (5) qs. to 10 ml.	1.0 $\mu\text{g.}/\mu\text{l.}$	0.5 $\mu\text{g.}/\mu\text{l.}$

1.0 ml. of Solution 1 was pipetted into a 2 ml. volumetric tube, to which was added 1.0 ml. of Cholestane Solution B. This procedure was continued for Solutions 2 to 6 until each tube contained a mixture of ethynodiol diacetate, mestranol, and cholestane. The final concentrations of ethynodiol diacetate, mestranol, and cholestane are shown on page 55.

2.0 $\mu\text{l.}$ of Solutions 1_B to 6_B were consecutively injected into the gas chromatograph with identical conditions to those used for chlormadinone acetate. When all three peaks of a solution had

Final Concentrations of Ethynodiol Diacetate, Mestranol, and Cholestane

<u>Solution</u>	<u>Ethynodiol Diacetate and Mestranol</u>		<u>Cholestane</u>		<u>Concentration</u>	
					<u>Ethynodiol Diacetate</u>	<u>Mestranol</u> <u>Cholestane</u>
1 _B	1.0 ml. Solution 1	+	1.0 ml. Solution B		10.0 $\mu\text{g.}/2\mu\text{l.}$	5.0 $\mu\text{g.}/2\mu\text{l.}$ 5.0 $\mu\text{g.}/2\mu\text{l.}$
2 _B	1.0 ml. Solution 2	+	1.0 ml. Solution B		8.0 $\mu\text{g.}/2\mu\text{l.}$	4.0 $\mu\text{g.}/2\mu\text{l.}$ 5.0 $\mu\text{g.}/2\mu\text{l.}$
3 _B	1.0 ml. Solution 3	+	1.0 ml. Solution B		6.0 $\mu\text{g.}/2\mu\text{l.}$	3.0 $\mu\text{g.}/2\mu\text{l.}$ 5.0 $\mu\text{g.}/2\mu\text{l.}$
4 _B	1.0 ml. Solution 4	+	1.0 ml. Solution B		4.0 $\mu\text{g.}/2\mu\text{l.}$	2.0 $\mu\text{g.}/2\mu\text{l.}$ 5.0 $\mu\text{g.}/2\mu\text{l.}$
5 _B	1.0 ml. Solution 5	+	1.0 ml. Solution B		2.0 $\mu\text{g.}/2\mu\text{l.}$	1.0 $\mu\text{g.}/2\mu\text{l.}$ 5.0 $\mu\text{g.}/2\mu\text{l.}$
6 _B	1.0 ml. Solution 6	+	1.0 ml. Solution B		1.0 $\mu\text{g.}/2\mu\text{l.}$	0.5 $\mu\text{g.}/2\mu\text{l.}$ 5.0 $\mu\text{g.}/2\mu\text{l.}$

emerged on the chromatogram, the next solution was injected.

Attenuation was adjusted accordingly.

(iii) Calibration Curve for Norethynodrel and Mestranol

The procedure employed in the preparation of a calibration curve for norethynodrel and mestranol was identical to that used in Part (i), Gas-liquid Chromatography, for chlormadinone acetate and mestranol. All aliquots were made to volume with chloroform before continuing dilution.

Stock Solution and Dilutions for Norethynodrel and

Mestranol

<u>Solution</u>		<u>Concentration</u>	
		<u>Norethynodrel</u>	<u>Mestranol</u>
1	0.500 g. Norethynodrel plus 0.250 g. Mestranol qs. to 25 ml.	20.0 $\mu\text{g.}/\mu\text{l.}$	10.0 $\mu\text{g.}/\mu\text{l.}$
2	8.0 ml. (1) qs. to 10 ml.	16.0 $\mu\text{g.}/\mu\text{l.}$	8.0 $\mu\text{g.}/\mu\text{l.}$
3	6.0 ml. (1) qs. to 10 ml.	12.0 $\mu\text{g.}/\mu\text{l.}$	6.0 $\mu\text{g.}/\mu\text{l.}$
4	4.0 ml. (1) qs. to 10 ml.	8.0 $\mu\text{g.}/\mu\text{l.}$	4.0 $\mu\text{g.}/\mu\text{l.}$
5	5.0 ml. (4) qs. to 10 ml.	4.0 $\mu\text{g.}/\mu\text{l.}$	2.0 $\mu\text{g.}/\mu\text{l.}$
6	5.0 ml. (5) qs. to 10 ml.	2.0 $\mu\text{g.}/\mu\text{l.}$	1.0 $\mu\text{g.}/\mu\text{l.}$
7	5.0 ml. (6) qs. to 10 ml.	1.0 $\mu\text{g.}/\mu\text{l.}$	0.5 $\mu\text{g.}/\mu\text{l.}$

500 $\mu\text{l.}$ of Solution 1 was pipetted into a 1 ml. volumetric tube, to which was added 500 $\mu\text{l.}$ of Cholestane Solution B. This procedure was continued for Solutions 2 to 7 until each tube contained a mixture of norethynodrel, mestranol, and cholestane. The final concentrations of norethynodrel, mestranol and cholestane are shown on page 57.

Final Concentrations of Norethynodrel, Mestranol, and Cholestane

Solution	Norethynodrel and Mestranol		Cholestane		Concentration	
					Norethynodrel	Mestranol
1 _B	500 μ l. Solution 1	+	500 μ l. Solution B		20.0 μ g./2 μ l.	10.0 μ g./2 μ l.
2 _B	500 μ l. Solution 2	+	500 μ l. Solution B		16.0 μ g./2 μ l.	8.0 μ g./2 μ l.
3 _B	500 μ l. Solution 3	+	500 μ l. Solution B		12.0 μ g./2 μ l.	6.0 μ g./2 μ l.
4 _B	500 μ l. Solution 4	+	500 μ l. Solution B		8.0 μ g./2 μ l.	4.0 μ g./2 μ l.
5 _B	500 μ l. Solution 5	+	500 μ l. Solution B		4.0 μ g./2 μ l.	2.0 μ g./2 μ l.
6 _B	500 μ l. Solution 6	+	500 μ l. Solution B		2.0 μ g./2 μ l.	1.0 μ g./2 μ l.
7 _B	500 μ l. Solution 7	+	500 μ l. Solution B		1.0 μ g./2 μ l.	0.5 μ g./2 μ l.

5.0 μ g./2 μ l.5.0 μ g./2 μ l.5.0 μ g./2 μ l.5.0 μ g./2 μ l.5.0 μ g./2 μ l.5.0 μ g./2 μ l.5.0 μ g./2 μ l.

2.0 μ l. of Solutions 1_B to 7_B were consecutively injected into the gas chromatograph with conditions of: column temperature, 222^o; injection port temperature, 244^o; detector temperature, 228^o; helium carrier gas at a flow of 30 ml./min.; air flow, 25 ml./min.; and hydrogen flow, 15 ml./min.. When all three peaks of a solution had emerged on the chromatogram the next solution was injected. Attenuation was adjusted accordingly.

(iv) Calibration Curve for Norethindrone and Mestranol

The procedure employed in the preparation of a calibration curve for norethindrone and mestranol was identical to that used in Part (i), Gas-liquid Chromatography, for chlormadinone acetate and mestranol. All aliquots were made to volume with chloroform before continuing dilution.

Stock Solution and Dilutions for Norethindrone and
Mestranol

<u>Solution</u>		<u>Concentration</u>	
		<u>Norethindrone</u>	<u>Mestranol</u>
1	0.250 g. Norethindrone plus 0.125 g. Mestranol qs. to 25 ml.	10.0 μ g./ μ l.	5.0 μ g./ μ l.
2	8.0 ml. (1) qs. to 10 ml.	8.0 μ g./ μ l.	4.0 μ g./ μ l.
3	6.0 ml. (1) qs. to 10 ml.	6.0 μ g./ μ l.	3.0 μ g./ μ l.
4	4.0 ml. (1) qs. to 10 ml.	4.0 μ g./ μ l.	2.0 μ g./ μ l.
5	5.0 ml. (4) qs. to 10 ml.	2.0 μ g./ μ l.	1.0 μ g./ μ l.
6	5.0 ml. (5) qs. to 10 ml.	1.0 μ g./ μ l.	0.5 μ g./ μ l.

1.0 ml. of Solution 1 was pipetted into a 2 ml. volumetric tube, to which was added 1.0 ml. of Cholestane Solution B, This

procedure was continued for Solutions 2 to 6 until each solution contained a mixture of norethindrone, mestranol, and cholestane. The final concentrations of norethindrone, mestranol, and cholestane are shown on page 60.

2.0 μ l. of Solutions 1_B to 6_B were consecutively injected into the gas chromatograph with conditions of: column temperature, 218^o; detector temperature, 222^o; helium carrier gas at a flow of 40 ml./min.; air flow, 25 ml./min.; and hydrogen flow, 15 ml./min.. When all three peaks of a solution had emerged on the chromatogram, the next solution was injected. Attenuation was adjusted accordingly.

D. Quantitative Assay of Pharmaceutical Preparations

An estimated number of oral contraceptive tablets required for a quintuplet assay were accurately weighed. After crushing the tablets with a pestle and mortar, an exact quantity of powder equal to a certain amount of steroid was added to a 10 ml. siliconized volumetric flask. The concentration of steroid was calculated to fall upon the prepared calibration curve.

About 5 ml. of chloroform was added, and the mixture was stirred magnetically for one hour. After complete dissolution of the steroids in the solution, the entire mixture was transferred to a 15 ml. siliconized centrifuge tube with adequate rinsing of the initial flask with chloroform. The tube was placed in the centrifuge and rotated at a speed of 1200 rpm for eight minutes. Using a siliconized Pasteur pipette, the upper solution was carefully removed and put into a siliconized 10 ml. round-bottomed flask, Centrifugation was repeated twice with two 2 ml. portions of

Final Concentrations of Norethindrone, Mestranol, and Cholestane

<u>Solution</u>	<u>Norethindrone and Mestranol</u>		<u>Cholestane</u>		<u>Concentration</u>	
					<u>Norethindrone</u>	<u>Mestranol</u> <u>Cholestane</u>
1 _B	1.0 ml. Solution 1	+	1.0 ml. Solution B	10.0 μ g./2 μ l.	5.0 μ g./2 μ l.	5.0 μ g./2 μ l.
2 _B	1.0 ml. Solution 2	+	1.0 ml. Solution B	8.0 μ g./2 μ l.	4.0 μ g./2 μ l.	5.0 μ g./2 μ l.
3 _B	1.0 ml. Solution 3	+	1.0 ml. Solution B	6.0 μ g./2 μ l.	3.0 μ g./2 μ l.	5.0 μ g./2 μ l.
4 _B	1.0 ml. Solution 4	+	1.0 ml. Solution B	4.0 μ g./2 μ l.	2.0 μ g./2 μ l.	5.0 μ g./2 μ l.
5 _B	1.0 ml. Solution 5	+	1.0 ml. Solution B	2.0 μ g./2 μ l.	1.0 μ g./2 μ l.	5.0 μ g./2 μ l.
6 _B	1.0 ml. Solution 6	+	1.0 ml. Solution B	1.0 μ g./2 μ l.	0.5 μ g./2 μ l.	5.0 μ g./2 μ l.

chloroform and the quantities of chloroform removed from the centrifuge tube were mixed together in the round-bottomed flask. With the use of the vacuum pump, the chloroform solution was evaporated to dryness. The steroid residue was dissolved in 500 μ l. of chloroform, 500 μ l. of 5.0 μ g./ μ l. Cholestane solution was added (Cholestane Solution B), and the flask was shaken manually. An immediate gas chromatographic analysis followed. Chromatographic conditions were identical to those used in preparation of the appropriate calibration curve.

The entire assay was repeated five times for each oral contraceptive. This procedure was applied to the trade preparations of Enovid-E [®], Ovulen [®] 0.5 mg., C-Quens [®], and Ortho-Novum [®] SQ.

TABLE VIII

Trade Preparations AssayedA. Combination Tablets

<u>Trade Name</u>	<u>Manufacturer</u>	<u>Component Steroids</u>
1. Enovid-E [®]	Searle	each tablet: 2.5 mg. Norethynodrel with 0.1 mg. Mestranol
2. Ovulen [®] 0.5 mg.	Searle	each tablet: 0.5 mg. Ethynodiol Diacetate with 0.1 mg. Mestranol

B. Sequential Tablets

<u>Trade Name</u>	<u>Manufacturer</u>	<u>Component Steroids</u>
1. C-Quens [®]	Lilly	15 white tablets: 0.08 mg. Mestranol 5 peach tablets: 2 mg. Chlormadinone Acetate with 0.08 mg. Mestranol
2. Ortho-Novum [®] SQ	Ortho	14 white tablets: 0.08 mg. Mestranol 6 blue tablets: 2 mg. Norethindrone with 0.08 mg. Mestranol

RESULTS AND DISCUSSION

I. INFRARED SPECTROPHOTOMETRY

Theory

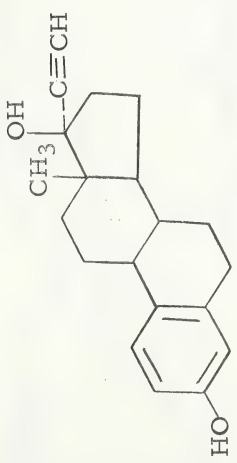
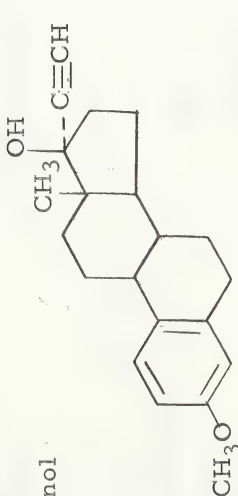
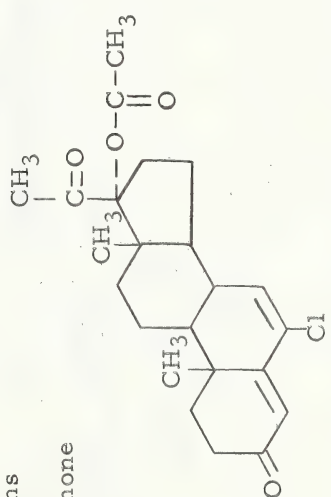
Scientific literature dealing with the infrared spectroscopy of organic compounds continues to expand at a very rapid rate. The current editions of the USP and the BP suggest the extensive use of this method as a means of identifying a large number of substances and preparations. Those assays related to the steroids studied in this research project are discussed in the Introduction. Many pharmaceutical preparations have also been qualitatively identified or quantitatively assayed by means of IR spectrophotometry.

Table IX lists the steroid structure of each estrogen and progestogen selected for study and the absorption chosen for the quantitative analysis of the pure steroid. Figure 1 illustrates that portion of the infrared spectrum selected for the analysis of the pure estrogens, ethinyl estradiol and mestranol. Figure 2 shows the selected areas of interest in the infrared for the pure steroid progestogens, chlormadinone acetate, dimethisterone, ethynodiol diacetate, megestrol acetate, norethin -
drone, norethindrone acetate, norethynodrel, and d-norgestrel (as dl-racemate).

To be a useful spectrophotometric solvent a liquid should show little infrared absorption. Chloroform was selected as the solvent of choice because it did not shown any absorption in the region used for the quantitative analysis of the steroids.

TABLE IX

Absorption Peak Area Selected for Quantitative Analysis

Compound	Structural Formula	Frequency in CHCl_3 (cm^{-1})	Structure Assignment (vibration of)
I. Estrogens			
1. Ethinyl Estradiol		a) 1605*, 1575 (shoulder)	aromatic ring A $\text{C}=\text{C}$ stretching
2. Mestranol		a) 1605*, 1570 (shoulder)	aromatic ring A $\text{C}=\text{C}$ stretching
II. Progestogens			
1. Chlormadinone Acetate		a) 1732* b) 1715 (shoulder) c) 1658 d) 1603, 1585 (shoulder)	C_{17} acetate carbonyl stretching C_{17} ketone carbonyl stretching α, β -unsaturated Δ^4 -3-ketone carbonyl stretching Δ^4, δ -diene-3-ketone $\text{C}=\text{C}$ stretching

Continued . . .

TABLE IX - Continued

Compound	Structural Formula	Frequency in CHCl_3 (cm^{-1})	Structure Assignment (vibration of)
2. Dimethisterone		a) 1658* b) 1600	α, β -unsaturated Δ^4 - 3-ketone carbonyl stretching Δ^4 -3-ketone $\text{C}=\text{C}$ stretching
3. Ethynodiol Diacetate		a) 1735* b) 1660 (shoulder)	C_3 and C_{17} acetate carbonyl stretching Δ^4 $\text{C}=\text{C}$ stretching
4. Megestrol Acetate		a) 1728 b) 1710 (shoulder) c) 1650* d) 1603, 1585	C_{17} acetate carbonyl stretching C_{17} ketone carbonyl stretching α, β -unsaturated Δ^4 - 3-ketone carbonyl stretching Δ^4, δ^6 -diene-3-ketone $\text{C}=\text{C}$ stretching

Continued . . .

TABLE IX - Continued

Compound	Structural Formula	Frequency in CHCl_3 (cm^{-1})	Structure Assignment (vibration of)
5. Norethindrone		a) 1660* b) 1615 (shoulder)	α, β -unsaturated Δ^4 - 3-ketone carbonyl stretching Δ^4 -3-ketone $\text{C}=\text{C}$ stretching
6. Norethindrone Acetate		a) 1740 b) 1663* c) 1615 (shoulder)	C_{17} acetate carbonyl stretching α, β -unsaturated Δ^4 - 3-ketone carbonyl stretching Δ^4 -3-ketone $\text{C}=\text{C}$ stretching
7. Norethynodrel		a) 1715*	$\Delta^5(10)$ -3-ketone carbonyl stretching
8. d-Norgestrel (as <u>dl</u> -racemate)		a) 1662* b) 1615 (shoulder)	α, β -unsaturated Δ^4 - 3-ketone carbonyl stretching Δ^4 -3-ketone $\text{C}=\text{C}$ stretching

* Exact peak selected for the analysis of the pure steroid.

Note: The structure assignments in this table were correlated with the published data contained in the reference "Chemical Applications of Infrared Spectroscopy," Academic Press, New York, and London, 1963, pages 406-477.

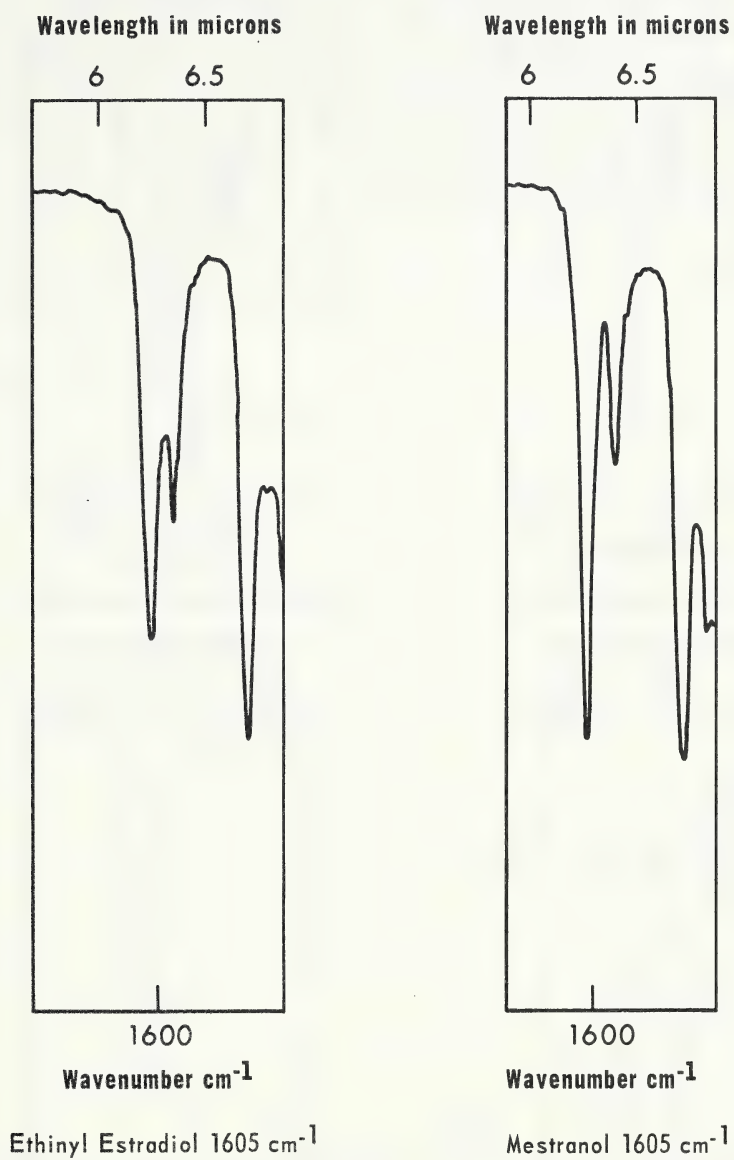


FIGURE 1: WAVELENGTH SELECTED FOR THE ANALYSIS OF THE PURE STEROID ESTROGENS

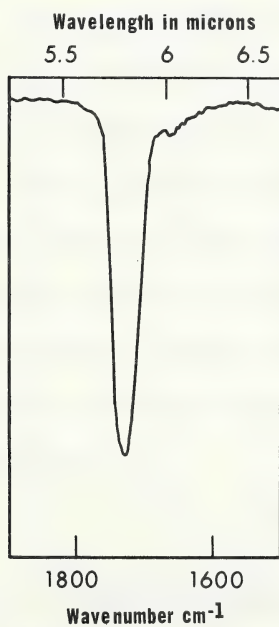
i) Chlormadinone Acetate 1732 cm^{-1} ii) Dimethisterone 1658 cm^{-1} iii) Ethynodiol Diacetate 1735 cm^{-1} iv) Megestrol Acetate 1650 cm^{-1}

FIGURE 2: WAVELENGTH SELECTED FOR THE ANALYSIS OF THE PURE STEROID PROGESTOGENS

An infrared spectrum cannot commonly distinguish a pure sample from an impure sample. In general, however, the spectrum of a pure sample will have fairly sharp and well-resolved absorption bands. The spectrum of a crude preparation that contains many different kinds of molecules will display broad and poorly resolved absorption bands because of the many absorptions that are present. Materials of high molecular weight that contain many different kinds of functional groups generally give poor spectra.

Quantitative Analysis

In a quantitative infrared assay of a substance, it is preferable to obtain:

1. an isolated peak
2. a sharp symmetrical peak
3. a peak with a level base line

and, 4. a peak with medium absorbance (between 15 and 80 per cent transmission).

For quantitative analysis the best horizontal base line is chosen and drawn and another line is drawn perpendicular through the maximum of the selected peak as illustrated in Figure 3. Since transmission is not directly proportional to concentration, transmission must be converted to absorbance which is directly related to concentration.

$$A_s = \log_{10} \left(\frac{\%T_o}{\%T} \right)$$

where A_s is the absorbance of a substance

T_o is the transmission of the solvent

and, T is the transmission of the solution

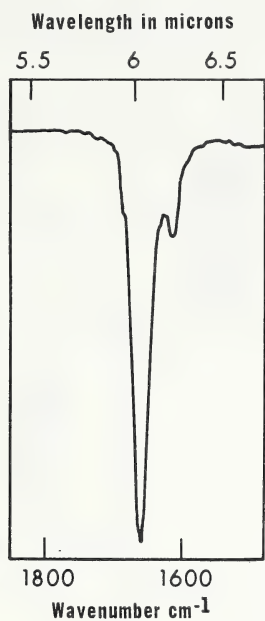
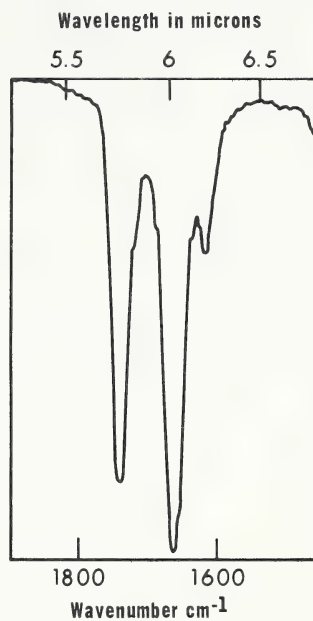
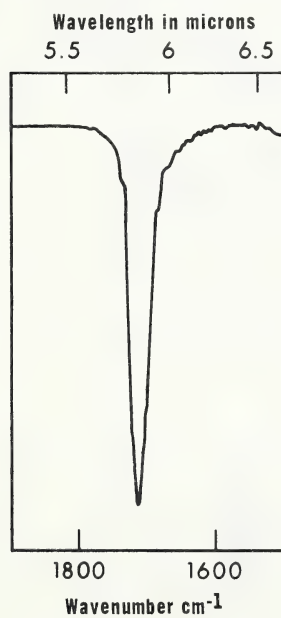
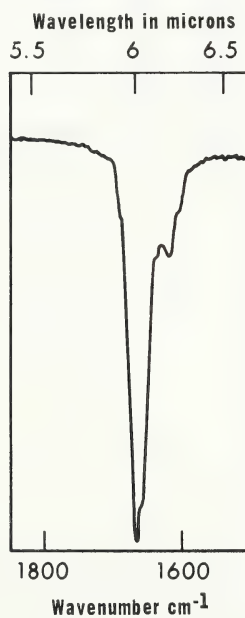
v) Norethindrone 1660 cm^{-1} vi) Norethindrone Acetate 1663 cm^{-1} vii) Norethynodrel 1715 cm^{-1} viii) d-Norgestrel (as dl-racemate) 1662 cm^{-1}

FIGURE 2 (continued) WAVELENGTH SELECTED FOR THE ANALYSIS OF THE PURE STEROID PROGESTOGENS

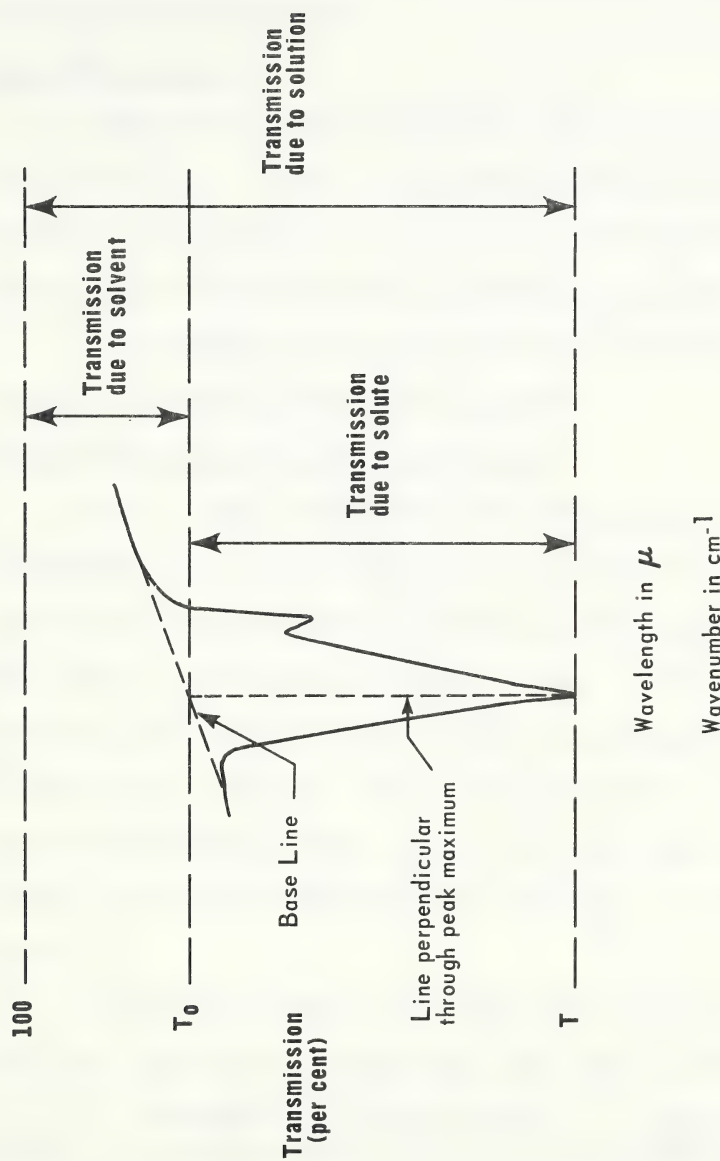


FIGURE 3: A TYPICAL REPRESENTATIVE PEAK SELECTED FOR QUANTITATIVE INFRARED ANALYSIS

From the calculated absorbance the Beer-Lambert relationship is obtained. Through the utilization of a calibration curve for a pure solute, the concentration of an unknown solution containing the pure solute can be read directly.

Calibration Curves

Calibration curves for the pure steroids are presented in Figures 4 to 13. A linear relationship between concentration and absorbance was obtained for each steroid indicating that the Beer-Lambert law was valid for the concentrations selected.

A presentation of the data used in the preparation of calibration curves is contained in Table XIII (Appendix).

Assay of Pharmaceutical Dosage Forms

The procedure employed in the determination of steroid concentration in a chloroform solution of an oral contraceptive trade preparation was identical to the procedure used in the preparation of the corresponding calibration curve. The transmission of the same peak used for the pure steroid was measured and converted to absorbance. Using the value for the absorbance calculated from the observed analytical data, the exact concentration of steroid in the solution was read directly from the calibration curve of the pure solute.

The solution concentration obtained from the calibration curve was converted to concentration per tablet as follows:

$$\text{Potency} = \frac{\text{Weight of one average tablet} \times \text{Concentration reading from the calibration curve}}{\text{Weight of powdered sample}}$$

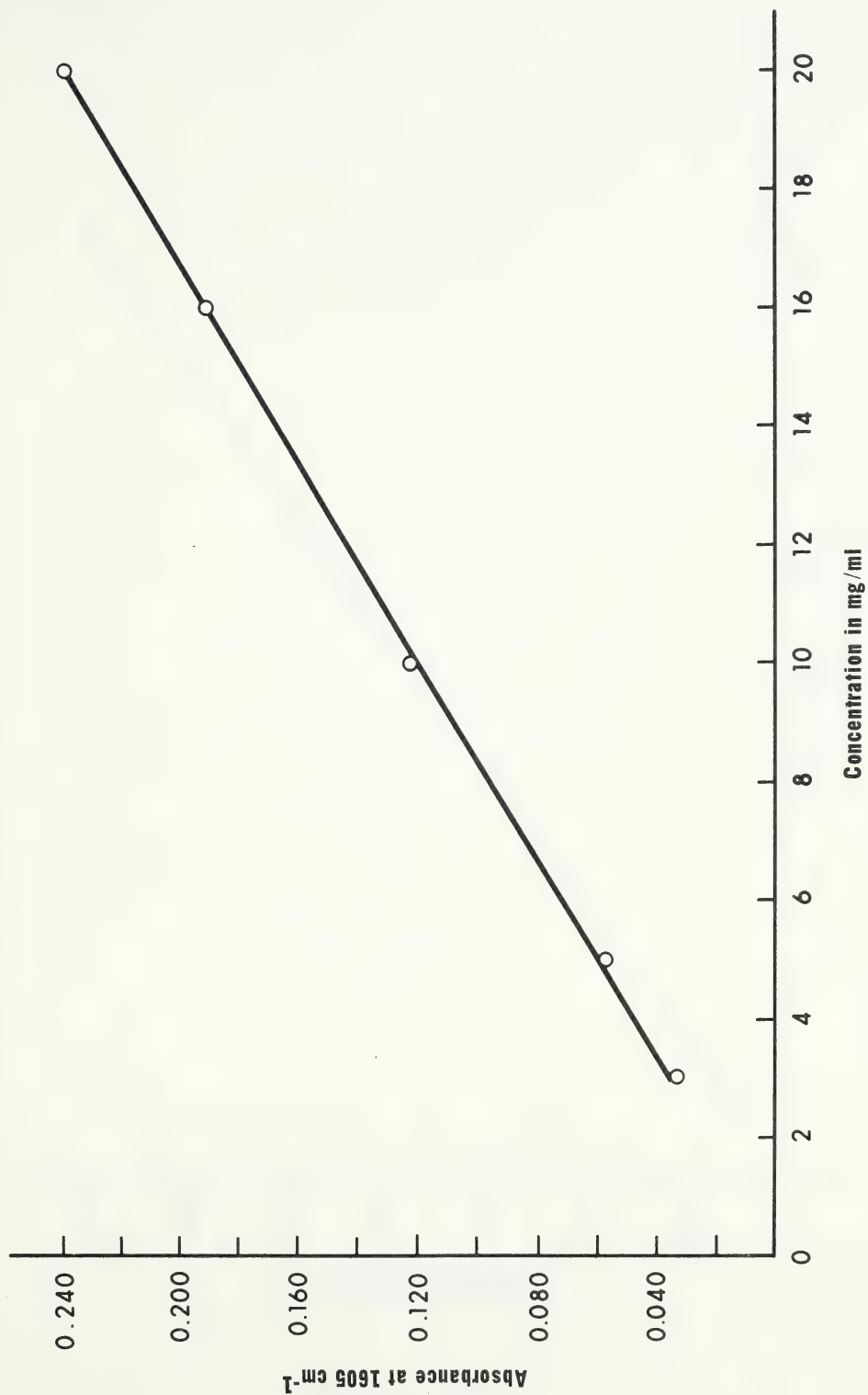


FIGURE 4: CALIBRATION CURVE FOR ETHINYL ESTRADIOL IN CHLOROFORM

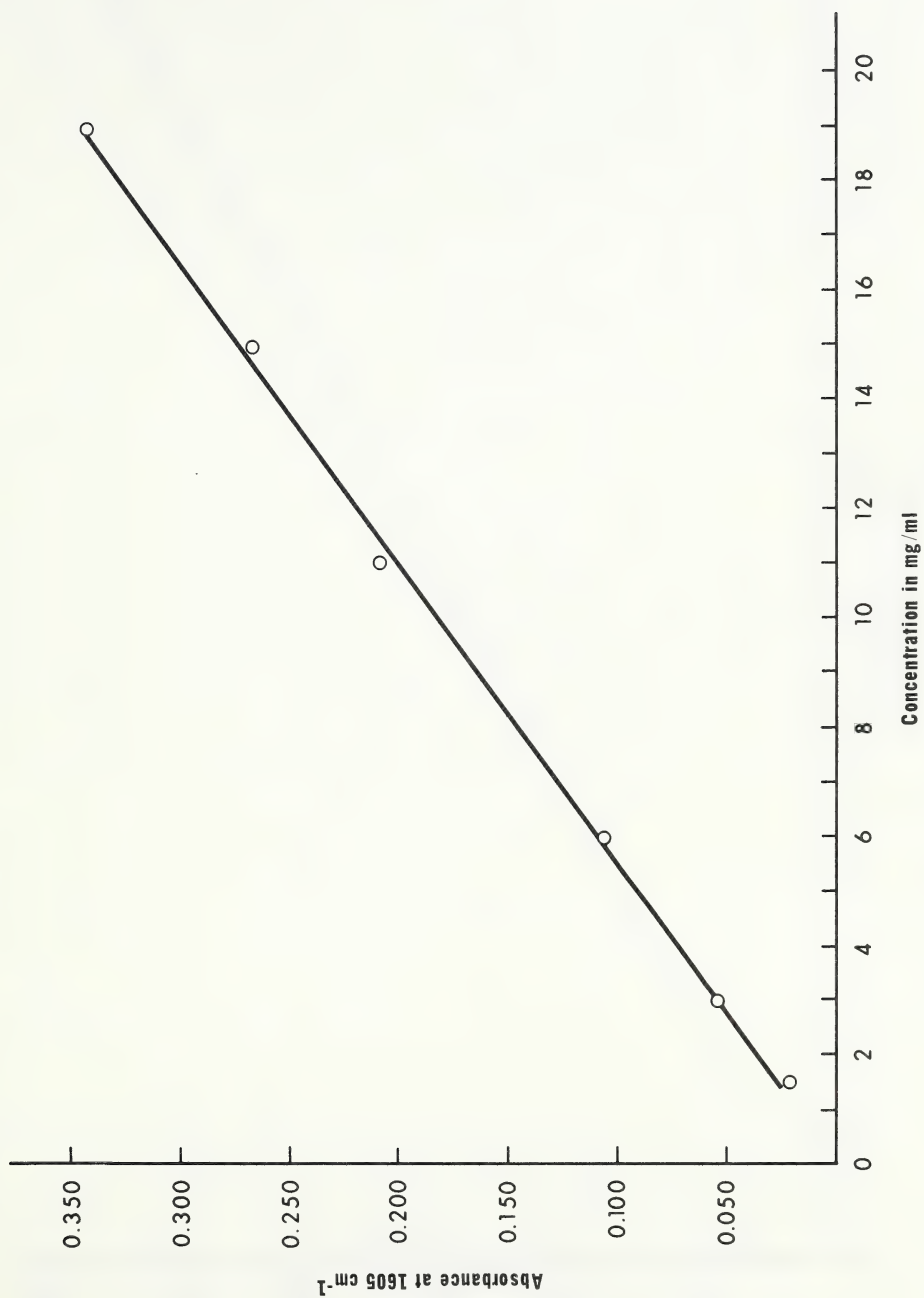


FIGURE 5: CALIBRATION CURVE FOR MESTRANOL IN CHLOROFORM

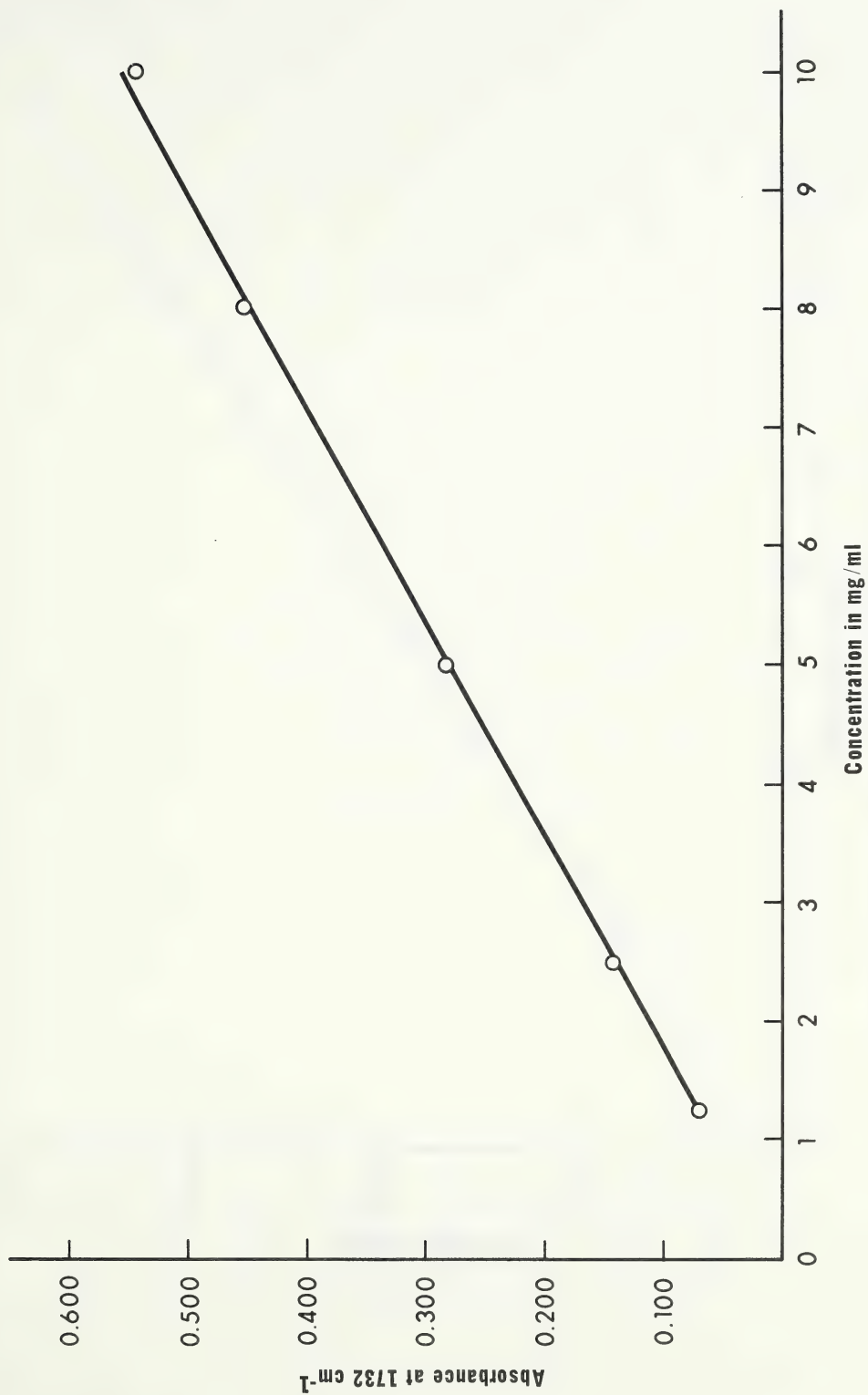


FIGURE 6: CALIBRATION CURVE FOR CHLORMADINONE ACETATE IN CHLOROFORM

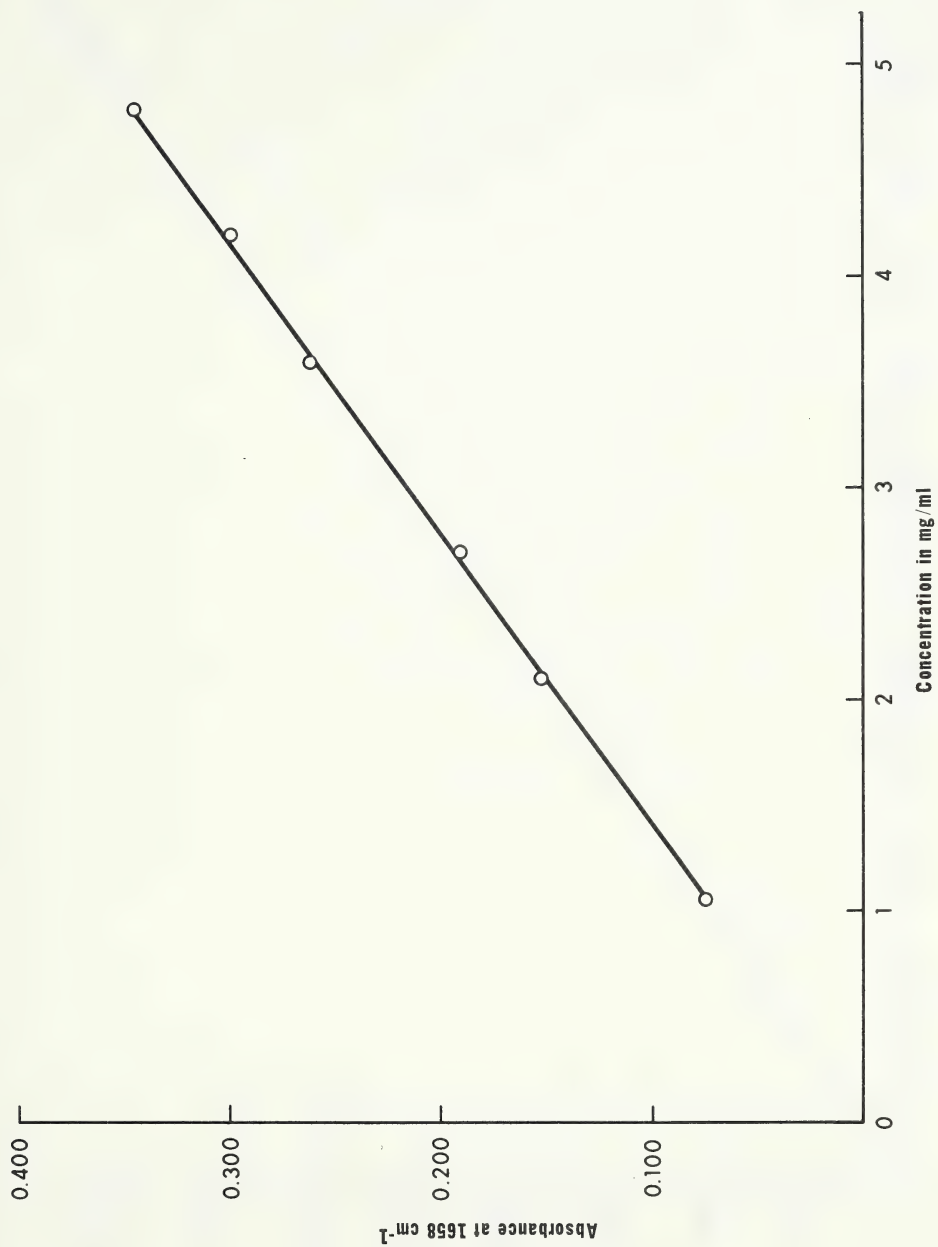


FIGURE 7: CALIBRATION CURVE FOR DIMETHISTERONE IN CHLOROFORM

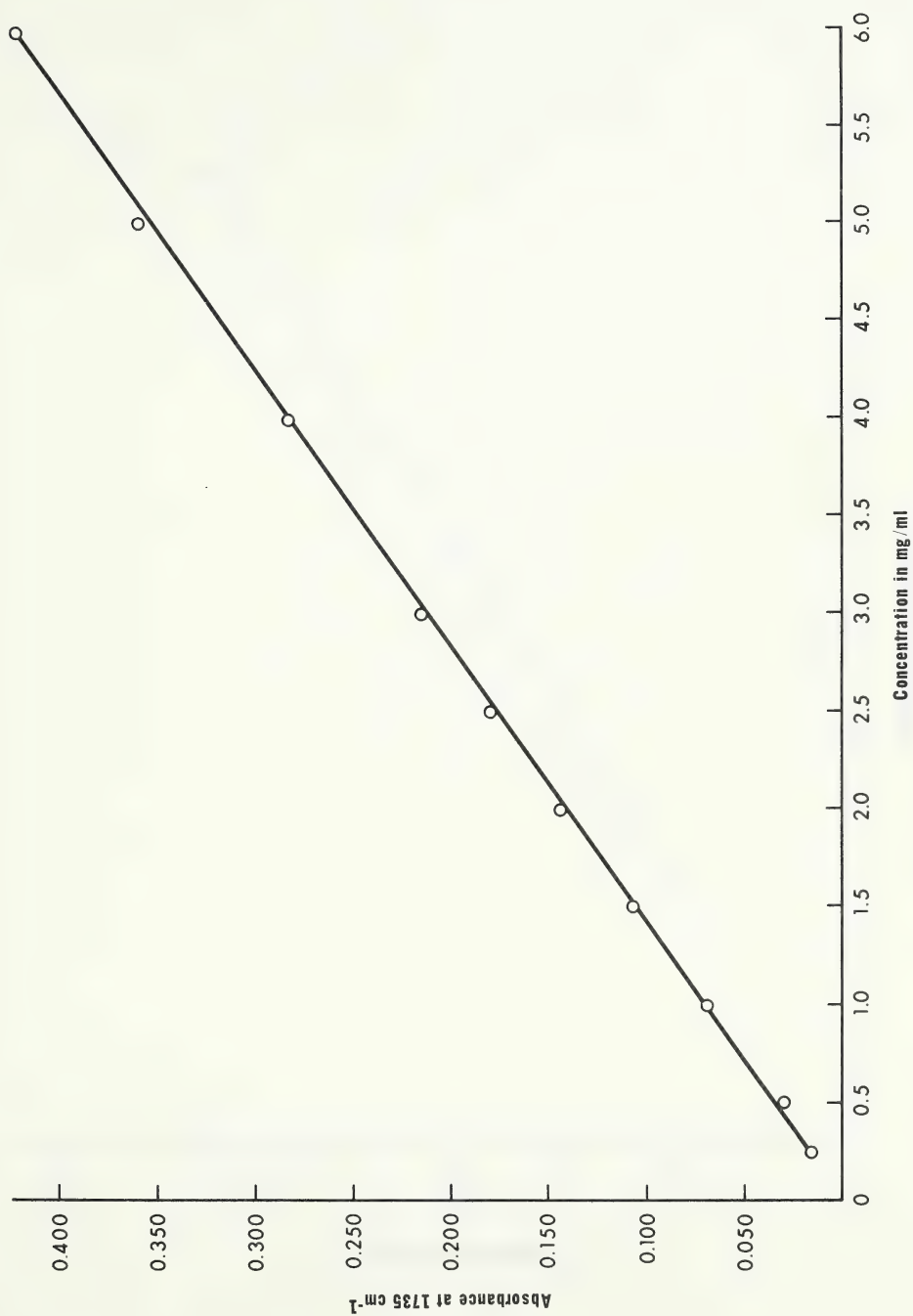


FIGURE 8: CALIBRATION CURVE FOR ETHYNOIDIOL DIACETATE IN CHLOROFORM

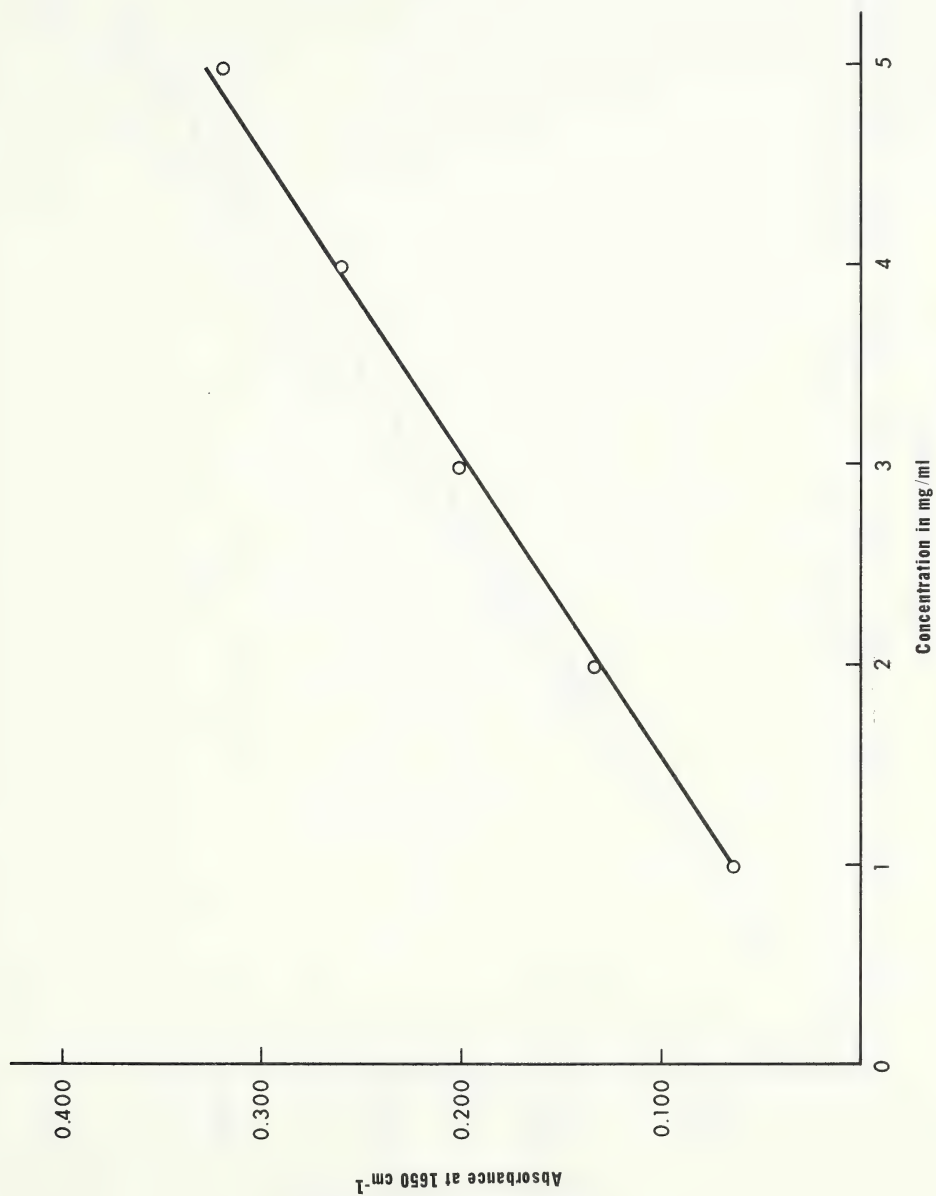


FIGURE 9: CALIBRATION CURVE FOR MEGESTROL ACETATE IN CHLOROFORM

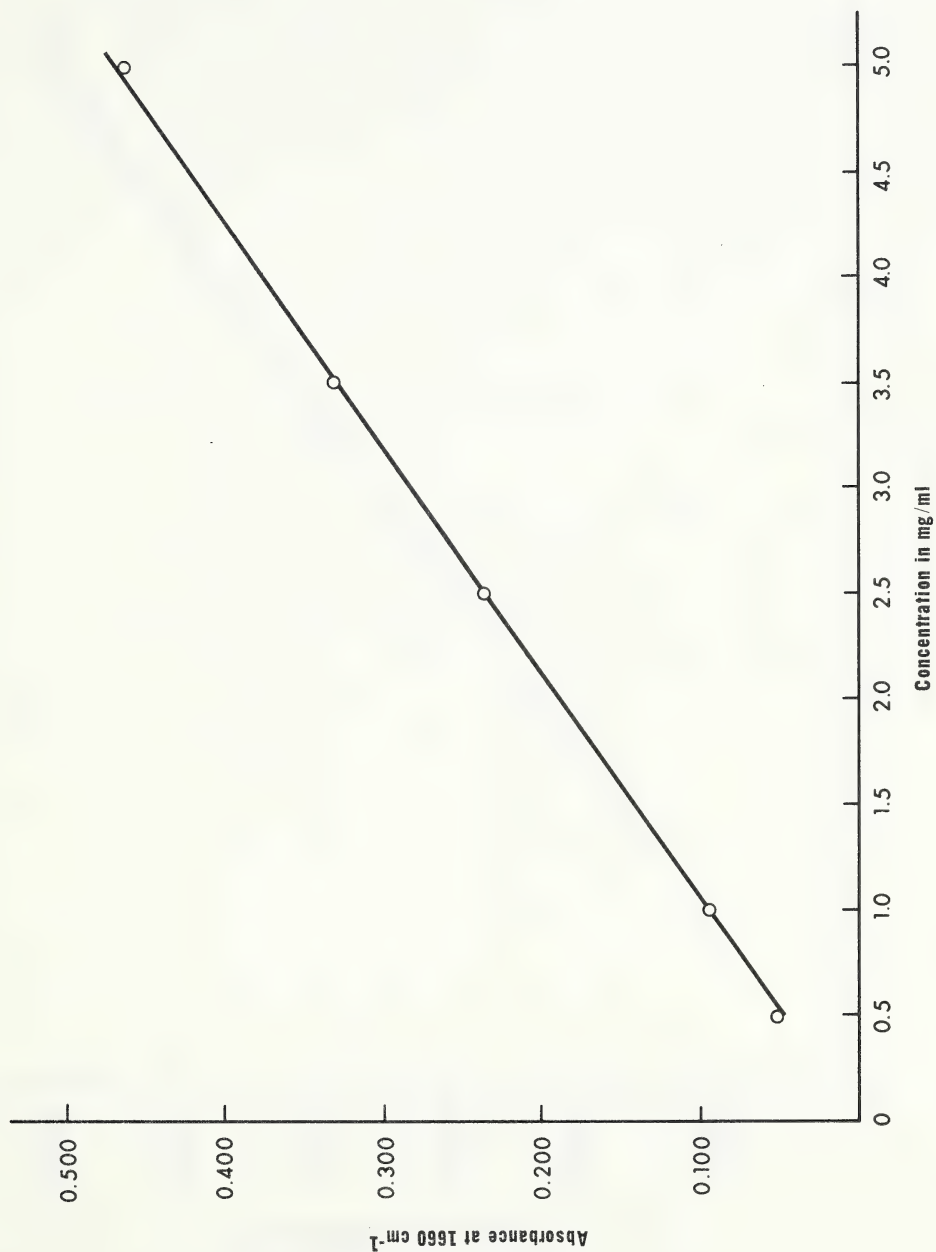


FIGURE 10: CALIBRATION CURVE FOR NORETHINDRONE IN CHLOROFORM

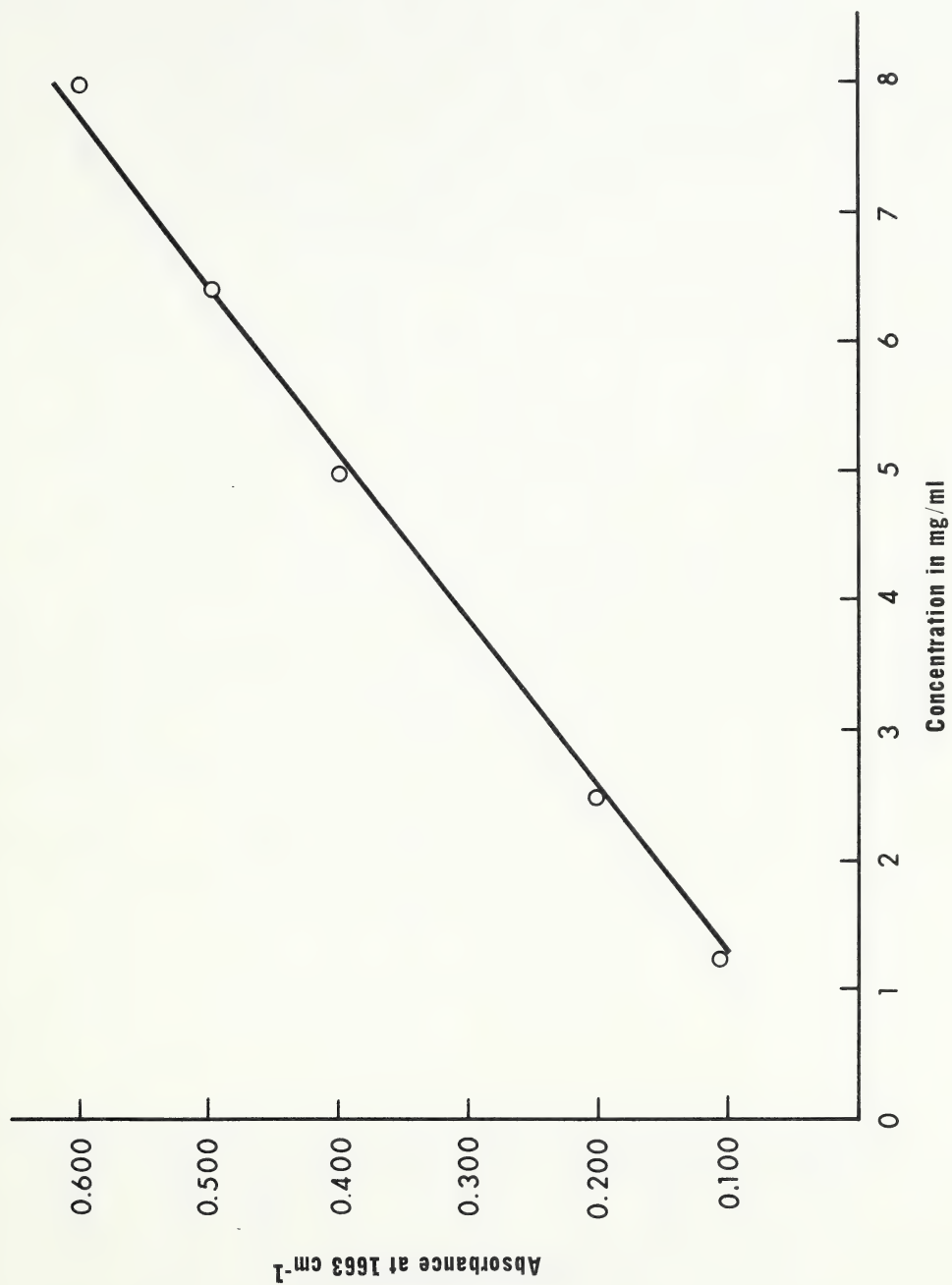


FIGURE 11: CALIBRATION CURVE FOR NORETHINDRONE ACETATE IN CHLOROFORM

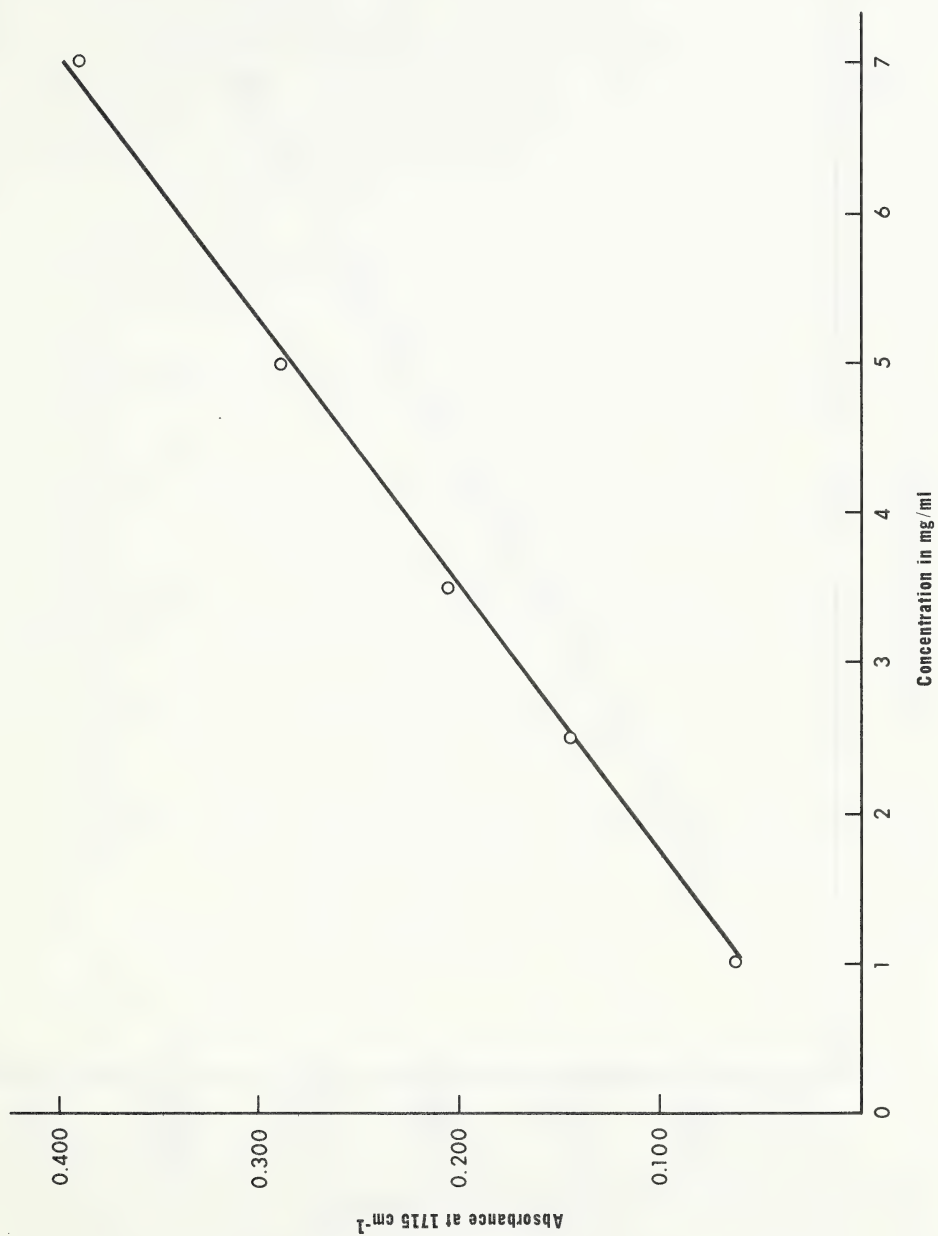


FIGURE 12: CALIBRATION CURVE FOR NORETHYNODREL IN CHLOROFORM

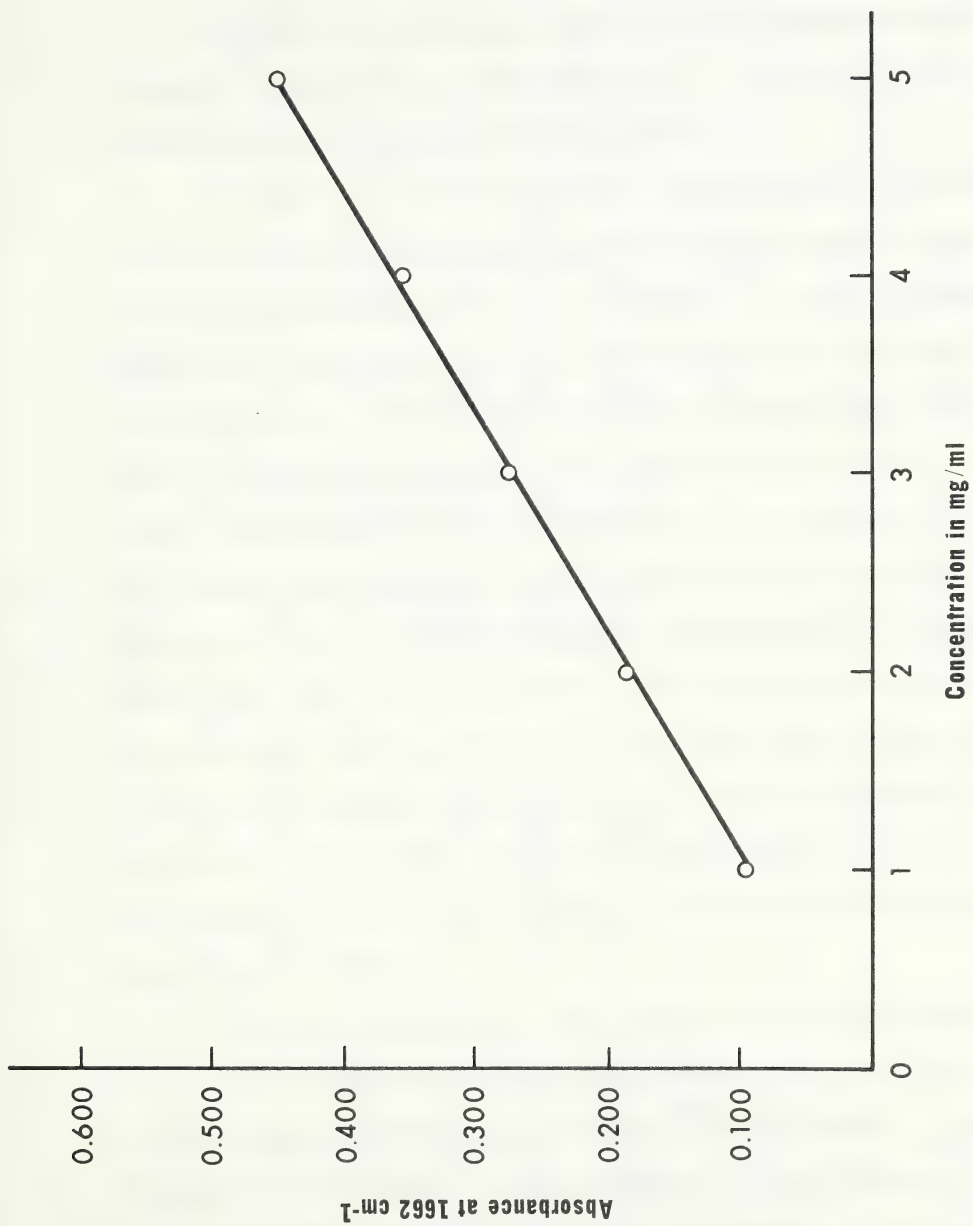


FIGURE 13: CALIBRATION CURVE FOR d-NORGESTREL (as dl-racemate) IN CHLOROFORM

The per cent recovery was calculated as:

$$\text{Per cent Recovery} = \frac{\text{Calculated potency per tablet}}{\text{Potency per tablet declared on label}} \times 100$$

The data for the assay of pharmaceutical dosage forms can be found in Tables XIV and XV (Appendix). A summary of this information is presented in Tables X and XI.

To check for possible absorption interference by the tablet diluents an infrared spectrum of each trade preparation, without the steroid components (placebo), was prepared. In some instances the manufacturer supplied a sample of the diluents for a particular trade preparation. The diluent materials included dyes, lubricants, glidants, and fillers prepared in the manufacturing process. For samples not obtained from the manufacturer, a representative sample was prepared by crushing a small quantity of the contraceptive tablet preparation. This was magnetically stirred in a chloroform solution for a few minutes and then collected by vacuum filtration. The procedure was repeated two or three times with fresh quantities of chloroform until the powder no longer contained the steroid components. All powder samples were thoroughly oven-dried at low temperature before their spectra were taken by the potassium bromide pellet method.

Figure 14 illustrates an oral contraceptive preparation whose diluents do not interfere with the infrared analysis in the area chosen for quantitative study (example, Ovulen[®] Three Week). Figure 15 illustrates an oral contraceptive preparation whose diluents do interfere with the infrared analysis in the area chosen for quantitative study (example, Norinyl[®] 2 mg.).

TABLE X

Assay of Pharmaceutical Dosage Forms

A. Combination Tablets

Trade Name	Steroid Analyzed	Per cent Recovery					Average ±One Standard Deviation
		Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	
1. Enovid [®] 5 mg.	Norethynodrel 5.0 mg.	97.0 96.4	98.8 96.0	98.2 101.0	100.6 100.6	100.4 97.0	98.6 ± 1.9
2. Enovid [®] 10 mg.	Norethynodrel 9.85 mg.	102.1 100.6	102.8 99.8	100.2 102.2	96.1 96.1	96.8 98.8	99.5 ± 2.5
3. Enovid-E [®]	Norethynodrel 2.5 mg.	93.2 93.6	90.8 92.0	96.4 90.8	94.4 96.8	91.2 90.8	93.0 ± 2.3
4a. Norlestrin [®] 1.0 mg. (per 2.0 ml. solution)	Norethindrone Acetate 1.0 mg.	73.0 73.0	77.0 77.0	79.0 79.0	79.0 79.0	75.0 75.0	76.6 ± 2.5
4b. Norlestrin [®] 1.0 mg. (per 5.0 ml. solution)	Norethindrone Acetate 1.0 mg.	90.0 90.0	90.0 87.0	94.0 96.0	91.0 90.0	90.0 90.0	90.8 ± 2.5
5. Norlestrin [®] 2.5 mg.	Norethindrone Acetate 2.5 mg.	93.6 93.6	93.2 94.8	92.4 92.0	91.6 91.2	91.6 91.6	92.6 ± 1.2
6. Ovral [®]	d-Norgestrel (as dl-racemate) 0.25 mg.	104.0 104.0	104.0 104.0	100.0 100.0	108.0 108.0	104.0 104.0	104.0 ± 2.3
7. Ovulen [®] Three week	Ethinodiol Diacetate 1.0 mg.	105.0 101.0	100.0 98.0	104.0 104.0	102.0 100.0	100.0 102.0	101.6 ± 2.2

TABLE XI
Assay of Pharmaceutical Dosage Forms

B. Sequential Tablets

Trade Name	Steroid Analyzed	Per cent Recovery					Average ± One Standard Deviation
		Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	
1. Oracon [®]	Dimethisterone 25 mg.	94.1 93.0	99.6 100.3	94.9 95.2	91.2 93.0	94.0 95.1	95.0 ± 2.9
2. Ovex [®]	Megestrol Acetate 5.0 mg.	93.4 93.0	96.8 94.6	90.6 89.0	92.2 92.2	98.0 98.0	93.8 ± 3.1
3. Secrovin [®]	Dimethisterone 25 mg.	99.6 99.6	102.1 103.9	100.5 103.4	99.0 101.4	101.3 100.2	101.1 ± 1.6
4a. Serial 28 [®] (per 2.0 ml. solution)	Megestrol Acetate 1.0 mg.	67.0 68.0	69.0 68.0	67.0 68.0	64.0 63.0	66.0 65.0	66.5 ± 2.0
4b. Serial 28 [®] (per 5.0 ml. solution)	Megestrol Acetate 1.0 mg	74.0 72.0	- -	- -	- -	- -	73.0

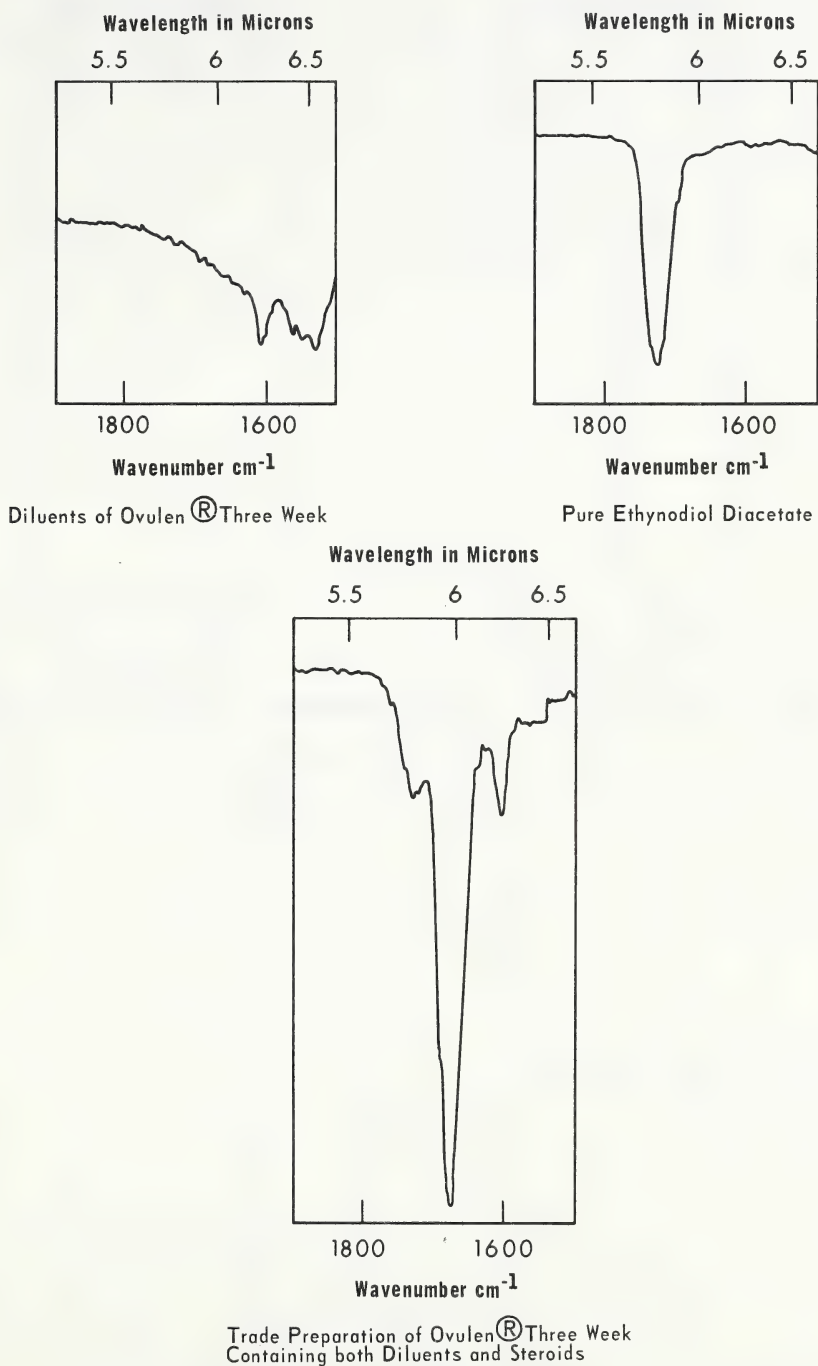


FIGURE 14: ONE EXAMPLE OF AN ORAL CONTRACEPTIVE PREPARATION WHOSE DILUENTS DO NOT INTERFERE WITH INFRARED ANALYSIS IN THE AREA SELECTED.

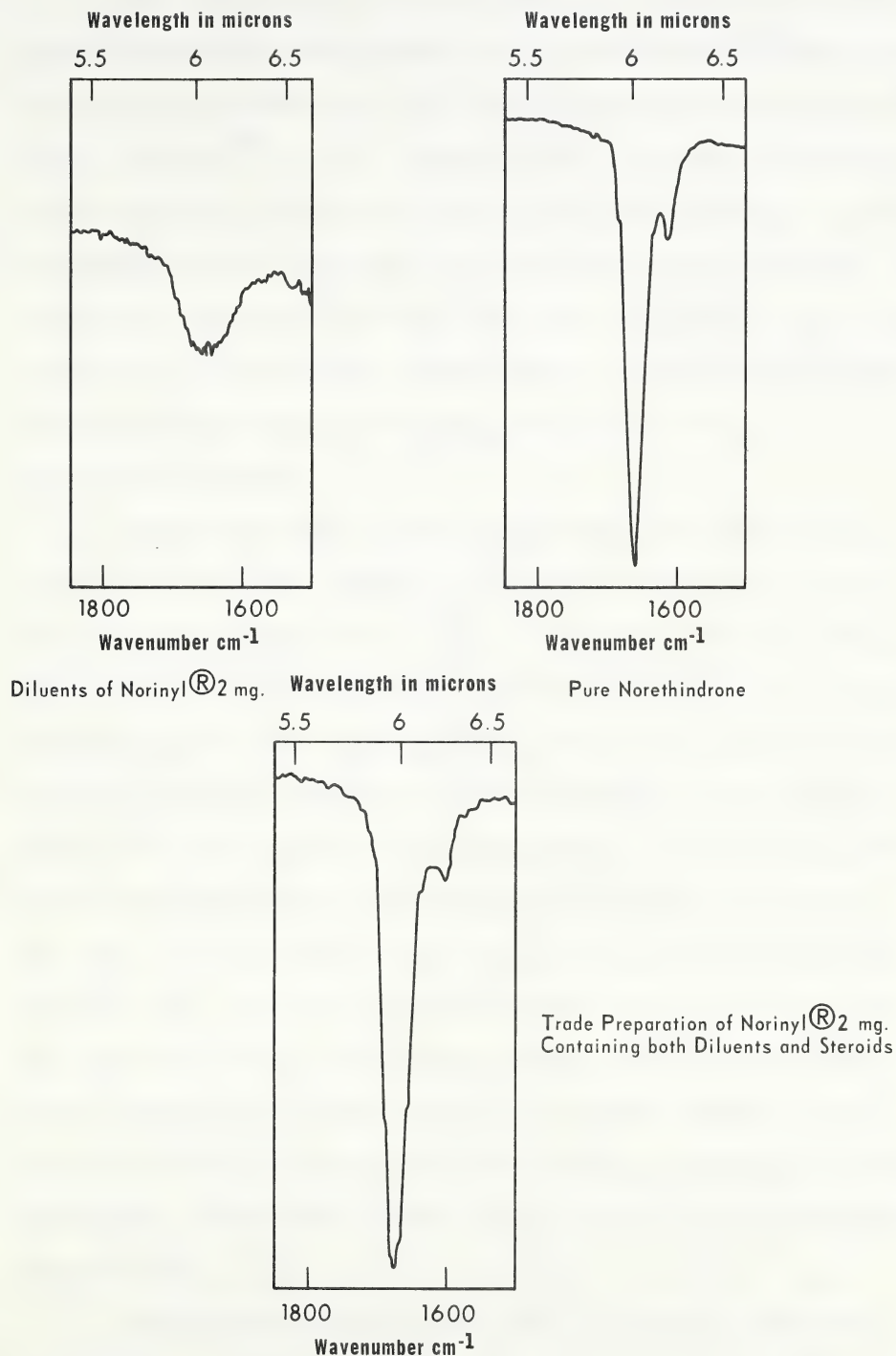


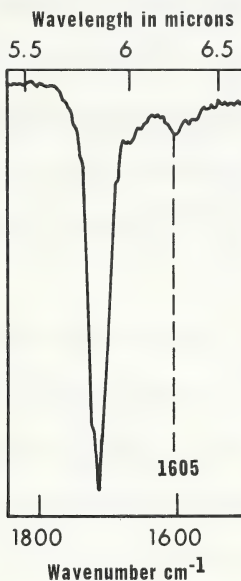
FIGURE 15: ONE EXAMPLE OF AN ORAL CONTRACEPTIVE PREPARATION WHOSE DILUENTS INTERFERE WITH INFRARED ANALYSIS IN THE AREA SELECTED.

The spectra of all diluent mixtures for the different trade preparations were similar except for that of C-Quens [®] (chlormadinone acetate and mestranol) and the Enovid preparations (Enovid [®] 5 mg., Enovid [®] 10 mg., Enovid-E [®]). In general the diluent spectra appear to contain absorption peaks for lactose (or sucrose), cornstarch, stearic acid and magnesium (or calcium) stearate. This conclusion was drawn from the comparison of spectra of pure powders commonly used in diluents and the spectra of the trade preparation placebos as received from the drug companies or those prepared as previously mentioned.

It was found that only the progestogen component of each trade preparation was suitable for quantitative infrared analysis. Since the progestogen was present in a concentration many times greater than that of the estrogen, the former gave a much stronger absorption peak when small quantities of the trade preparation were used for analysis. When the quantity of tablets was increased to about twice that used for the original analysis and maintaining the same volume of chloroform solution, the estrogen peak still did not show an absorption strong enough to be accurately measured (Figure 16a). When the quantity of tablets was increased to about ten times that used for the original analysis and maintaining the same volume of chloroform solution, the entire solution was too concentrated for quantitative infrared analysis, resulting in greatly overlapping peaks which trailed to about zero per cent transmission (Figure 16b).

In the infrared assay of the trade preparation containing chlormadinone acetate, the progestogen absorption peak was

- a. Through the utilization of twice the number of tablets in the same volume of solution.



Enovid® 5 mg.
The mestranol peak at 1605 cm^{-1}
does not give an absorption strong
enough to be accurately measured.

- b. Through the utilization of ten times the number of tablets in the same volume of solution.

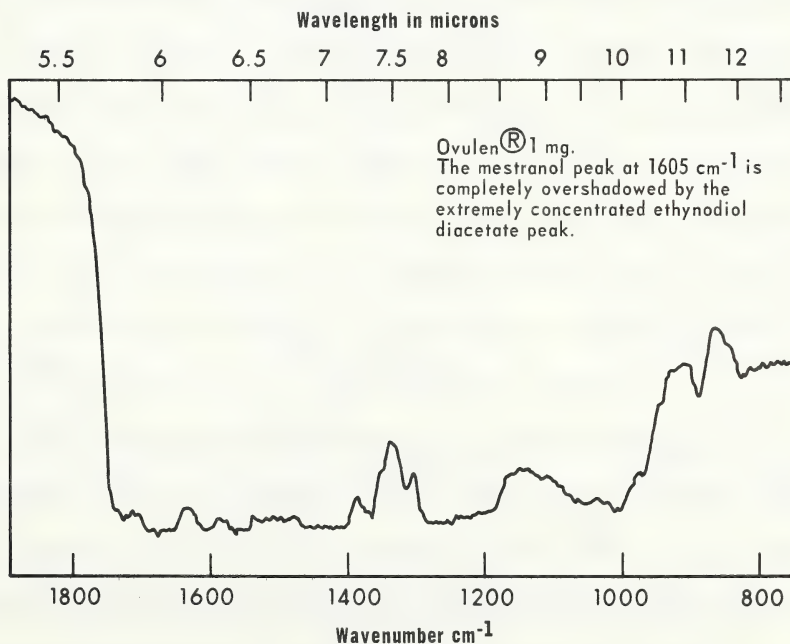


FIGURE 16: EXAMPLE OF AN ATTEMPT TO ANALYZE THE ESTROGEN COMPONENT OF A TRADE PREPARATION.

obscured by an overlapping diluent peak. The spectra obtained for the same preparation varied in appearance within a few minutes possibly indicating the occurrence of a chemical change (Figure 17). This reaction was accompanied by a thick deposit on the infrared cell. Consequently an accurate analytical measurement of the anti-fertility preparation C-Quens [®] was not possible using this procedure.

The infrared assay of the trade preparations containing dimethisterone, Oracon [®] and Secrovin [®], was more successful. Since the accompanying tablet diluents in a pharmaceutical preparation tend to change the base line from that of the pure steroid, a correction was made. Selecting approximately the same concentration of steroid in the spectra obtained for the preparation of the corresponding calibration curve, a horizontal line was drawn from a chosen point. A second line was drawn from the same point (base line) intersecting the perpendicular line rising from the peak. The angle between these lines was measured (Figure 18a), and an artificial base line was constructed using this measured angle (Figure 18b). The potency calculated from the analytical procedure closely corresponds with the manufacturer's claims for both Oracon [®] and Secrovin [®] (see Table XI, page 85).

For ethynodiol diacetate the angle drawn between the horizontal and base lines was 2.0° . When the base line correction was applied to the oral contraceptives Ovulen [®] 0.5 mg. and Ovulen [®] Three Week, the former preparation indicated some excipient interference. The ethynodiol diacetate absorption in the latter preparation was also broad and uneven but a definite peak was

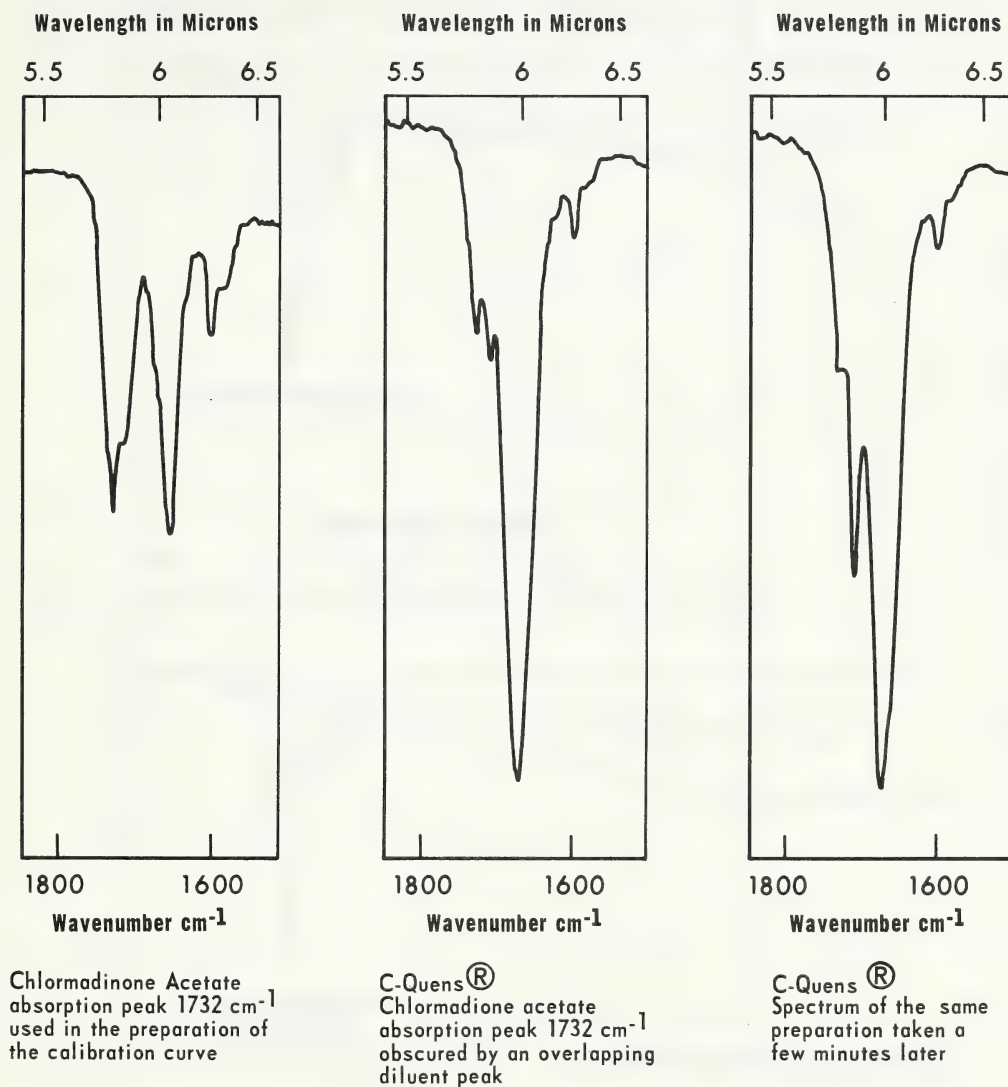


FIGURE 17: VARIATION IN THE INFRARED SPECTRA OF C-QUENS®

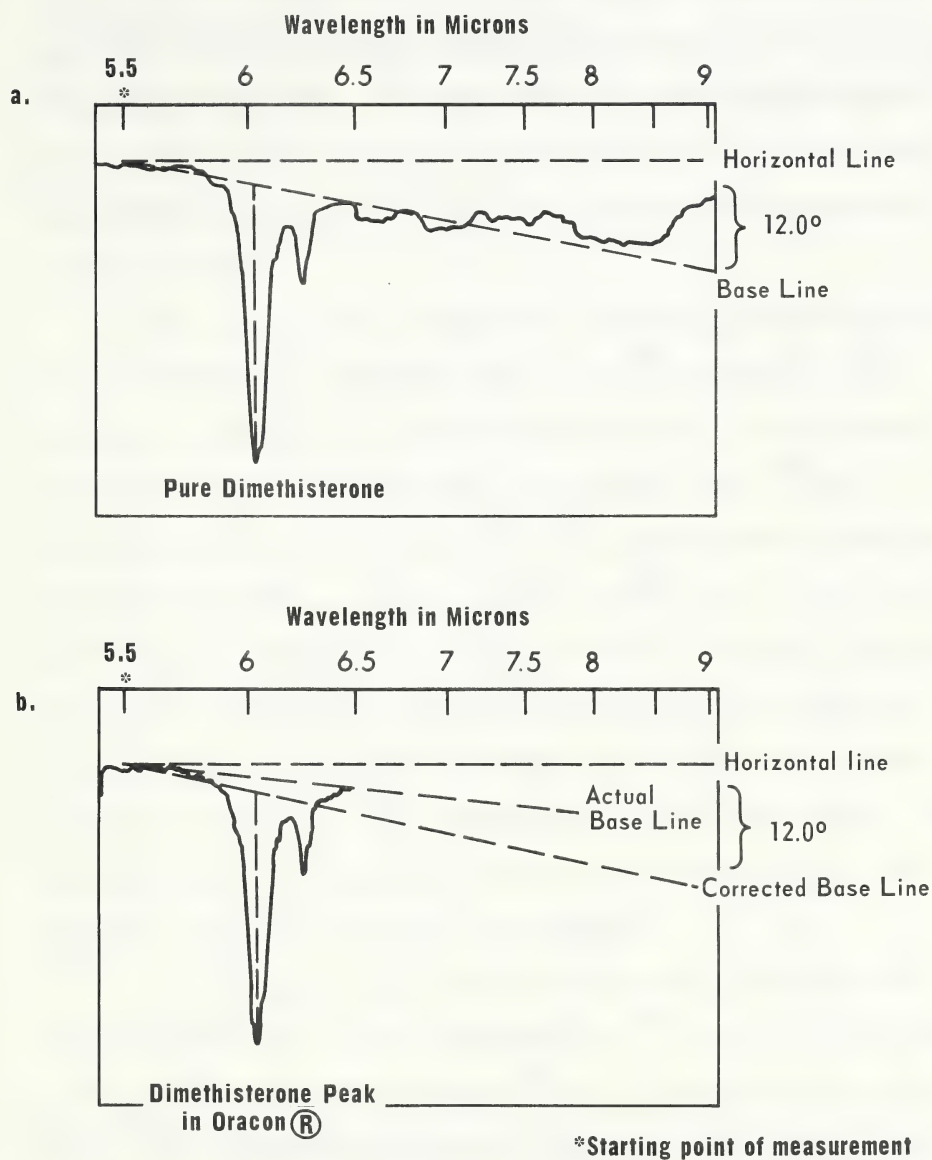


FIGURE 18: METHOD OF MEASURING TRANSMISSION IN THE PHARMACEUTICAL PREPARATIONS

visible (Figure 19). However, as the calculated potency average for Ovulen[®] Three Week was over 100 per cent (see Table X, page 84), there may also be diluent interference in the vicinity of the progestogen peak. Another difficulty in this measurement was the broad peak produced by pure ethynodiol diacetate rather than the ideal sharp and narrow peak required for quantitative infrared analysis.

The anti-fertility preparations containing megestrol acetate as the progestogen component were Ovex[®] and Serial 28[®]. By using the measured angle base line correction of 16.5° between horizontal and base lines of the pure steroid, Ovex[®] gave a calculated potency corresponding to the manufacturer's claim (see Table XI, page 85). However, Serial 28[®] gave a result much lower than the labelled potency (see Table XI, page 85), although the spectra of the preparation appear to have no interferences. By increasing both the number of tablets used in the assay and the volume of chloroform, the percentage recovery was raised. Perhaps the difficulty in this instance was the loss of product during the filtration of the minute volume of solution.

Norethindrone was present in the trade preparations of Norinyl[®] 1 mg., 2 mg., Ortho-Novum[®] 1 mg., 2 mg., 5 mg., Norquen[®], and Ortho-Novum[®] SQ. In each instance the 1660 cm^{-1} peak of norethindrone was completely covered by a much stronger 1670 cm^{-1} diluent absorption. Measurement by the developed analytical procedure was not possible in these preparations.

Norlestrin[®] 1.0 mg. and 2.5 mg. contained the progestogen norethindrone acetate. The measured angle of 6.5° calculated

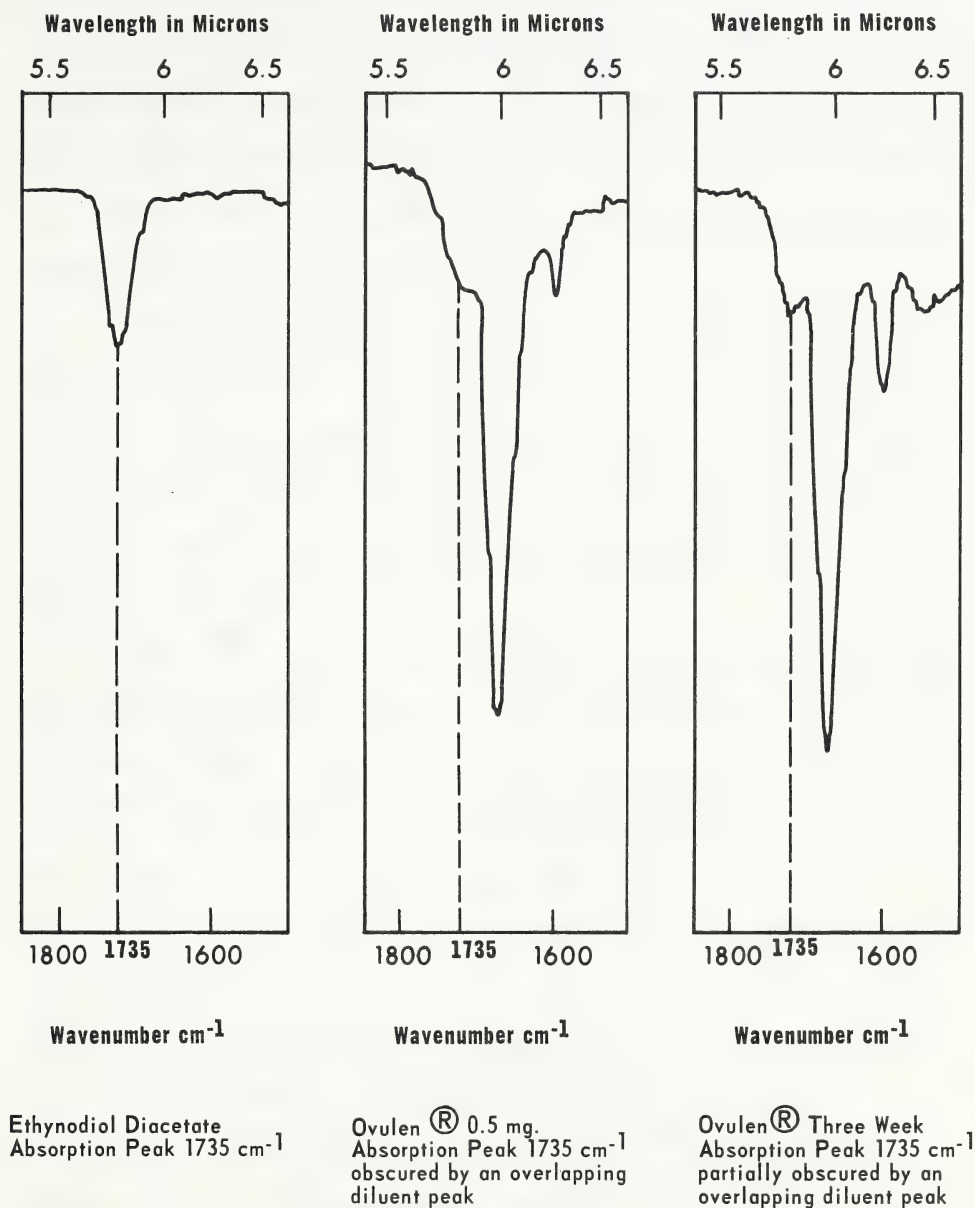


FIGURE 19: VARIATION IN THE INFRARED SPECTRA OF OVULEN
INCLUDING OVULEN® 0.5 mg. AND OVULEN® THREE WEEK

between the horizontal and base lines of the pure steroid was satisfactory when applied as a correction to the preparation with the greater potency but not when applied to Norlestrin [®] 1.0 mg. in a small volume of chloroform (2.0 ml.). When the same concentration was extracted with a larger volume of chloroform (5.0 ml.) the calculated potency increased to a value corresponding to the manufacturer's claim (see Table X, page 84).

The progestogen norethynodrel was contained in the preparations of Enovid [®]. Through the use of the measured angle 2.0° , as calculated between the horizontal and base lines of the pure steroid, all Enovid [®] tablets were quantitatively assayed to contain potencies corresponding to the manufacturer's claim (see Table X, page 84).

Ovral [®] was the only anti-fertility preparation containing d-norgestrel (as dl-racemate). Through the developed analytical procedure, using an angle of 3.0° obtained from pure d-norgestrel, Ovral [®] was found to contain an average potency of 104.0 ± 2.3 per cent progestogen (see Table X, page 84). This percentage recovery is slightly high, and may be attributed to one or both of the following reasons: (a) the absorption peak appears sharp and fairly narrow but some tablet excipient may be absorbing at the same wavelength thus increasing the calculated recovery of progestogen, (b) the average tablet of Ovral [®] is claimed to contain 0.25 mg. of d-norgestrel, which is only a fraction of the usual progestogen concentration contained in other trade preparations. During the manufacturing process there is quite likely some difficulty in procuring an even distribution of this small quantity of steroid

in a large quantity of placebo powder , resulting in individual tablets whose potency would either be slightly higher or lower than the declared amount. This particular problem would be minimized if the concentration of progestogen is increased, as in the case of all remaining anti-fertility preparations.

For most of the pharmaceutical products , the results obtained by the infrared quantitative method were in satisfactory agreement when compared with the results published by authors using other methods. The technique would seem to have a relatively high degree of accuracy and precision where no interference from excipient material occurred. While maintaining the concentration at approximately the same level, it appeared necessary to increase the volume of solution for those trade preparations containing a lower potency of progestogen in order to obtain an accurate analysis. In instances where overlapping of excipient and steroid peaks occurred, quantitative infrared analysis of the progestogen steroids was not possible and could not be performed unless some method could be developed where complete separation of placebo components is achieved. In this investigation chloroform does not always appear to be an ideal solvent , particularly in those cases where dissolution of interfering excipients occurs. These preparations include C-Quens[®] , Ovulen[®] 0.5 mg. , Norinyl[®] 1 mg. , 2 mg. , Ortho-Novum[®] 1 mg. , 2 mg. , 5 mg. , Norquen[®] , and Ortho-Novum[®] SQ. Another solution to this problem would be to select another peak for quantitative analysis where neither the solvent nor the excipients interfere.

II. GAS-LIQUID CHROMATOGRAPHY

Theory

The utilization of gas-liquid chromatography for the successful separation of steroids was first described by VandenHeuvel and associates in 1960. Since then, the technique has been widely used.

Although the principles involved in the GLC separation process of steroids are the same as those used for other classes of substances, somewhat different techniques are required to obtain optimum results. As the theoretical boiling points of steroids lie far above the temperature at which degradation begins, these compounds should not be expected to survive GLC treatment. In practice, however, GLC can be applied successfully on many compounds of high molecular weight at temperatures well below the region of thermal degradation, provided that the stationary phase has a low polarity and is used in small amounts. The use of concentrations between 0.5 and 3.0 per cent is normally satisfactory.

Depending on the volatility of the steroid, that is, with regard to its molecular weight and its number of functional groups, the column temperature used for analysis may range between 190 and 240°C. Usually the GLC of steroids is performed under isothermal conditions. Temperature programming is useful only in cases where the sample consists of compounds having an extensive range of retention times.

A wide variety of stationary phases has been used for the GLC of steroid mixtures. According to the dominant kind of stationary phase interaction with the sample, considerable

differences in selectivity between phases are observed. Unsubstituted methylpolysiloxanes and higher paraffins show no specific interactions with steroid functional groups, and on these phases the components of a steroid mixture are eluted in the order of their volatilities, according to the following approximate arrangement:



The stationary phase, OV-1, used in this research project falls into this category.

Among the technical difficulties that may occur in the practical application of GLC for the analysis of steroids is the partial or complete decomposition or adsorption of the samples on the column. This behavior appears to be strongly dependent upon the qualities of the materials which come into contact with the sample during the chromatographic procedure. The catalytic activity appears to be at a minimum when an all-glass apparatus is used.

Inert solid supports suitable for steroid separations are only those from which catalytically active impurities can be totally removed. Diatoport S (diatomaceous earth, silanized) used in this research project fulfilled this requirement. Silanization itself is not a remedy against catalytic effects, since these effects are caused mainly by the presence of metallic impurities, however silanization serves as a means to suppress the adsorptive properties of the pure support.

Quantitative Analysis

When applied to quantitative analysis gas-liquid chromatography can simultaneously separate minute quantities of several constituents with speed, sensitivity and accuracy. It is particularly

suitable for complex mixtures containing a number of structurally similar substances.

It is known that the area under a chromatographic peak or the height of a peak is proportional to the amount of substance present in the carrier gas, that is, to the quantity of solute required to produce each peak.

Measurement of peak height, as utilized in this research project, is useful in quantitative analysis only when all operating parameters can be reproduced exactly from one experiment to the next. Careful adjustment of carrier gas flow rate is particularly important, since peaks broaden and become smaller with increasing residence time on the column (Figure 25). Consequently, peak heights will vary with the retention times of the compound even when the detector response is approximately the same. Both relative and absolute peak heights increase with temperature even when the flow rate is kept constant; thus it is necessary to construct a calibration curve under the same conditions used for analysis. Peak height is employed mostly for the analysis of compounds with short retention times since the peaks are both sharp and symmetrical and large responses are obtained for comparatively minor changes in sample composition or sample concentration.

Calibration Method

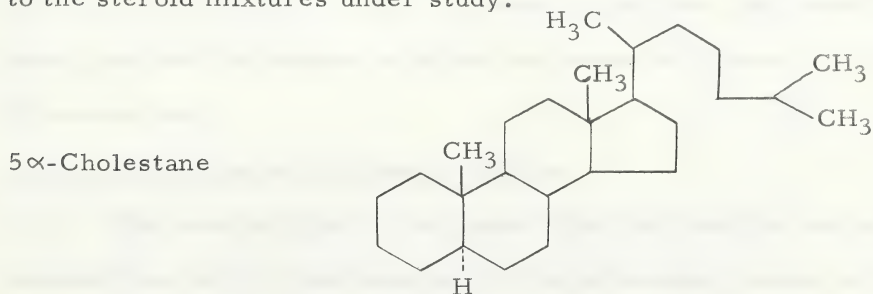
Calibration is required to ascertain the response of the detector-recorder system to the presence of a known amount of solute passing through the detector system.

One of the major problems in constructing a calibration curve is the introduction of an accurately measured sample into the

chromatograph. . Most syringes have only fair volume reproducibility. For this reason the internal standard method has been developed.

An internal standard should be selected with a retention time as close as possible to that of the peak or peaks being measured without overlap, and should have the same chemical character as the components of the mixture. If several widely spaced peaks are being quantitated, it is best to select a standard for each peak. If the retention times of two sample components are relatively close, the ideal situation would be to select a standard that peaks between them, if possible. The peak height of the standard should not differ to any extent from those of the components being measured. Generally, it is impractical to use internal standards when a large number of components are being measured because of excessive dilution of the sample and difficulty in finding materials with the requisite retention times. Internal standards are valuable in that they compensate for minor variations in operating parameters, particularly important when peak height is the basis for quantitation.

5 α -Cholestane was selected as the internal standard in this research project, initially because of its close structural similarity to the steroid mixtures under study.



With this technique a known amount of cholestane is added to a known volume of a solution to be quantitated. The two (or more)

compounds are mixed and are thus present in the solution in a set weight ratio. If the solvent evaporates, the concentration of the two or more solutes will increase to the same degree. Also, when the sample is injected, component loss is theoretically the same for all substances.

In applying the internal standard method to quantitative analysis, the peak height of the steroid being measured is divided by the peak height of cholestane. The initial concentration of the compound being assayed is determined from a plot of the ratios obtained with solutions containing constant concentrations of the internal standard and known varying amounts of the steroid being quantitated. For accurate quantitation it is desirable that the calibration curve determined by this method be linear.

Steroid calibration curves prepared by this technique are shown in Figures 20 to 22b. The data used to prepare the calibration curves is presented as a summary in Table XVI (Appendix).

Figure 23 illustrates a typical chromatogram for chlormadinone acetate, mestranol and cholestane mixtures. Typical chromatograms for ethynodiol diacetate, mestranol and cholestane mixtures, norethynodrel, mestranol and cholestane mixtures, and norethindrone, mestranol and cholestane mixtures are shown in Figures 24, 25 and 26 respectively.

Assay of Pharmaceutical Dosage Forms

The peak heights of component steroids from the extracted tablets (Gas-liquid Chromatography, Experimental), along with the internal standard, were measured in the same manner as for the preparation of the corresponding calibration curve. After the ratio

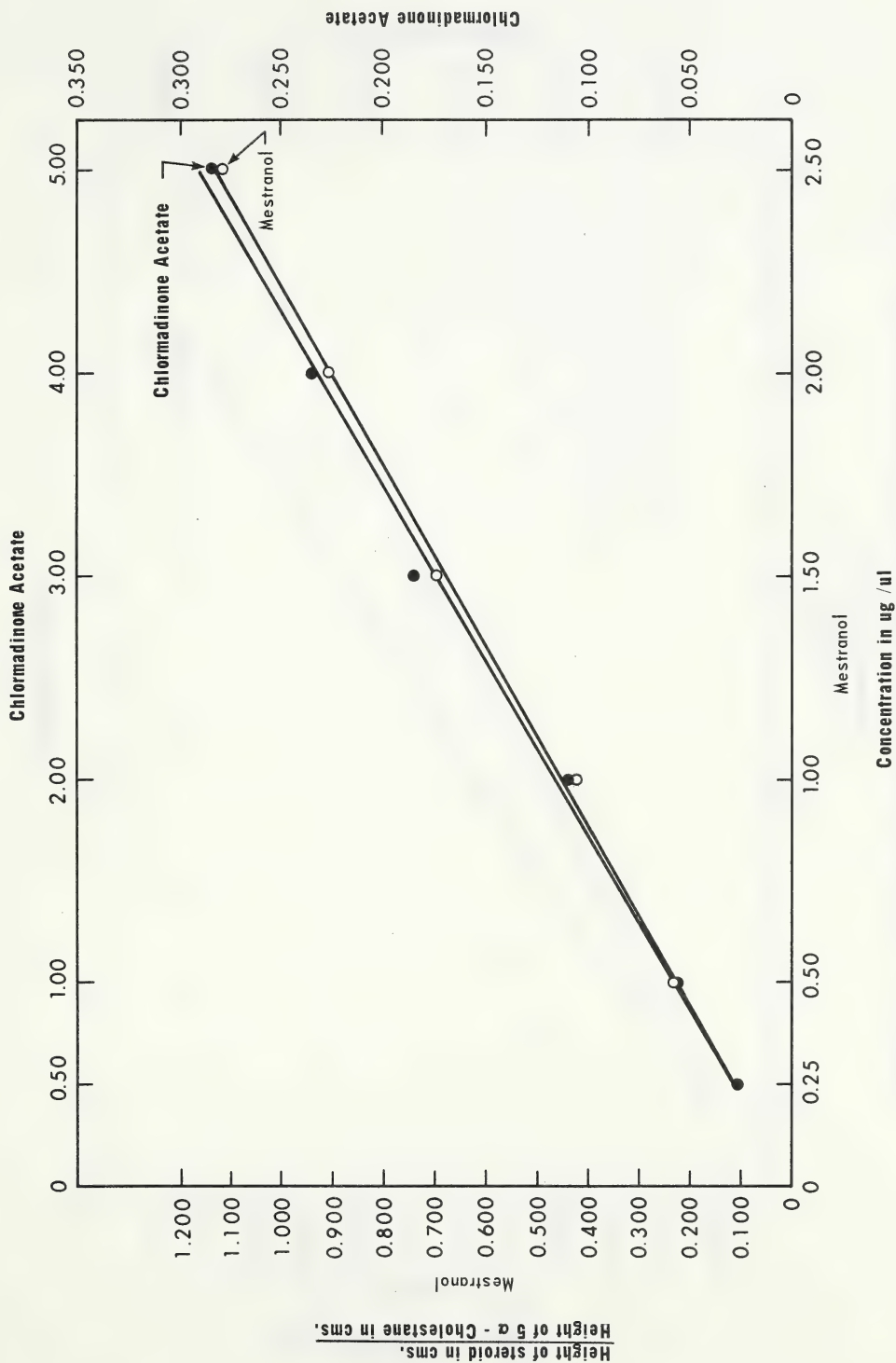


FIGURE 20: CALIBRATION CURVE FOR CHLORMADINONE ACETATE AND MESTRANOL IN CHLOROFORM

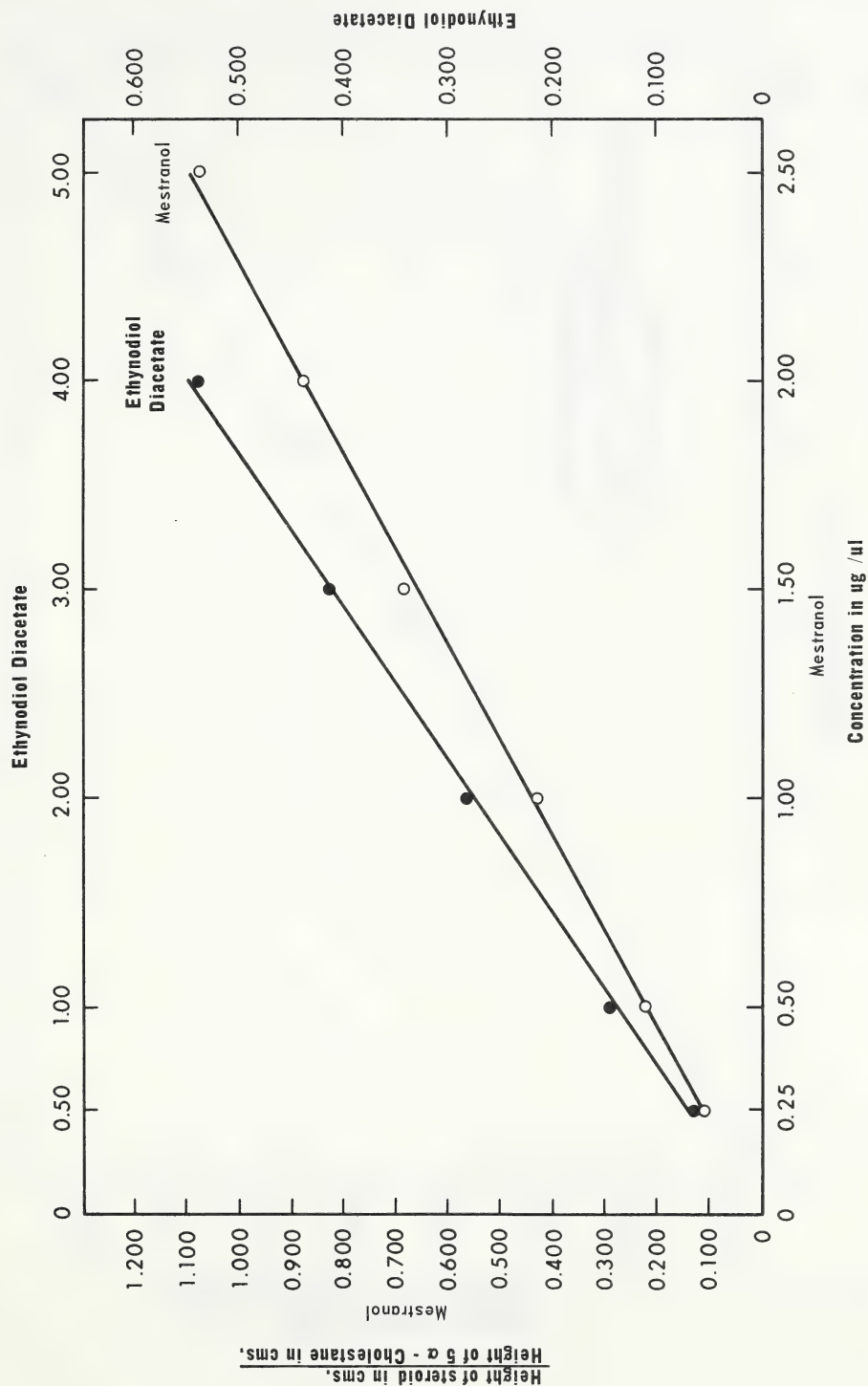


FIGURE 21: CALIBRATION CURVE FOR ETHYNODIOL DIACETATE AND MESTRANOL IN CHLOROFORM

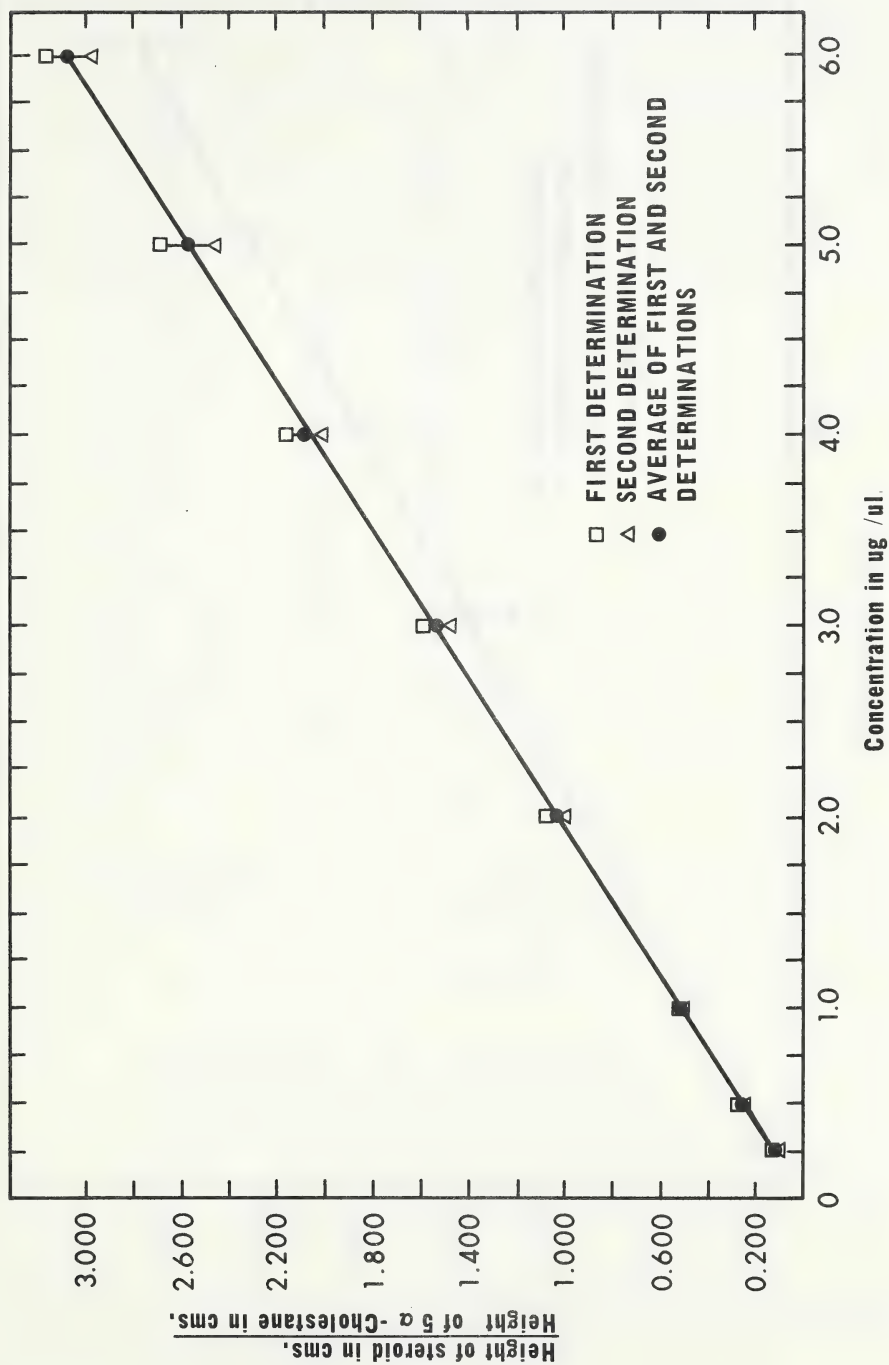


FIGURE 22a: CALIBRATION CURVE FOR MESTRANOL FROM THE NORETHYNODREL AND MESTRANOL SOLUTION MIXTURES.

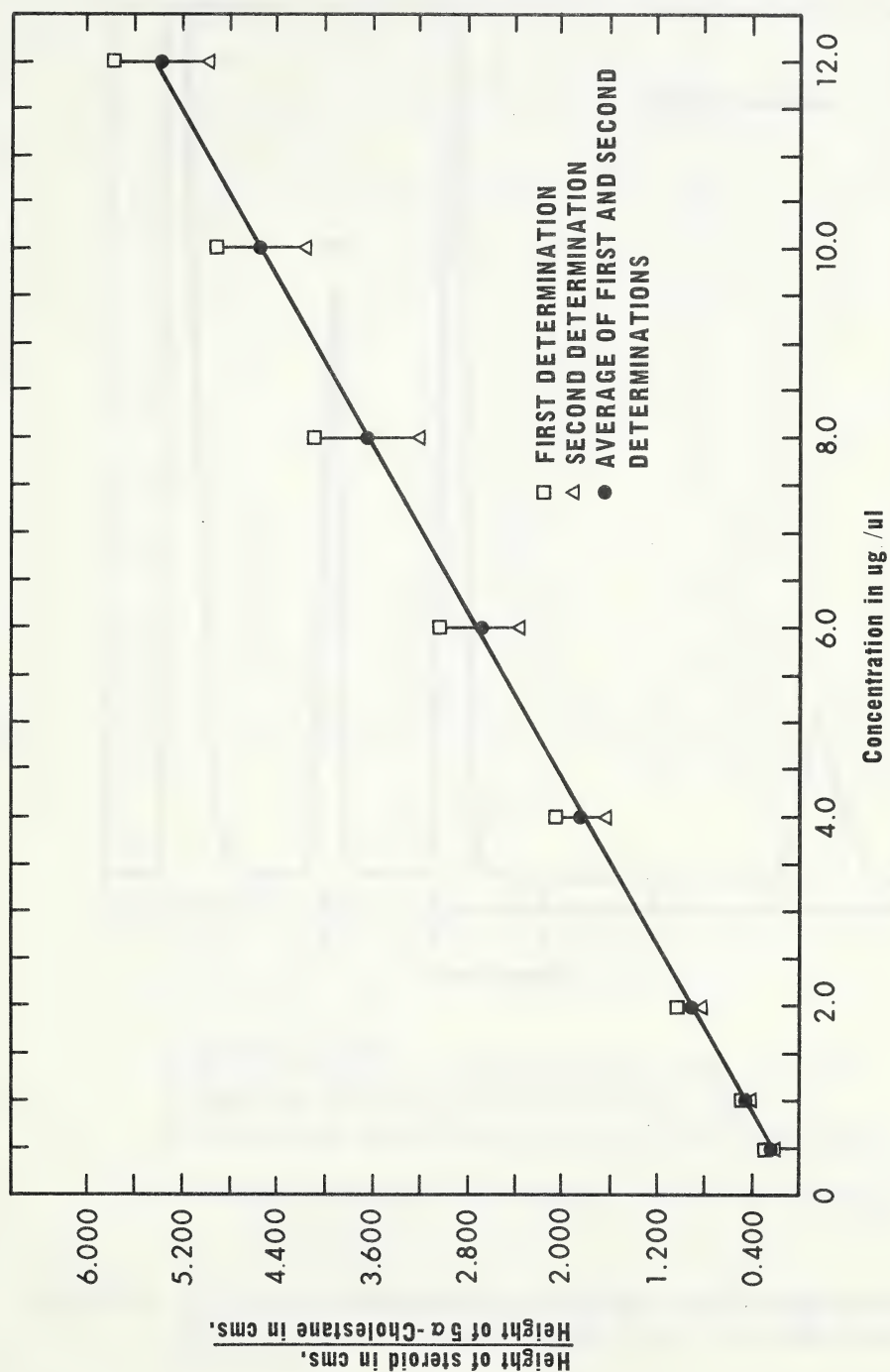
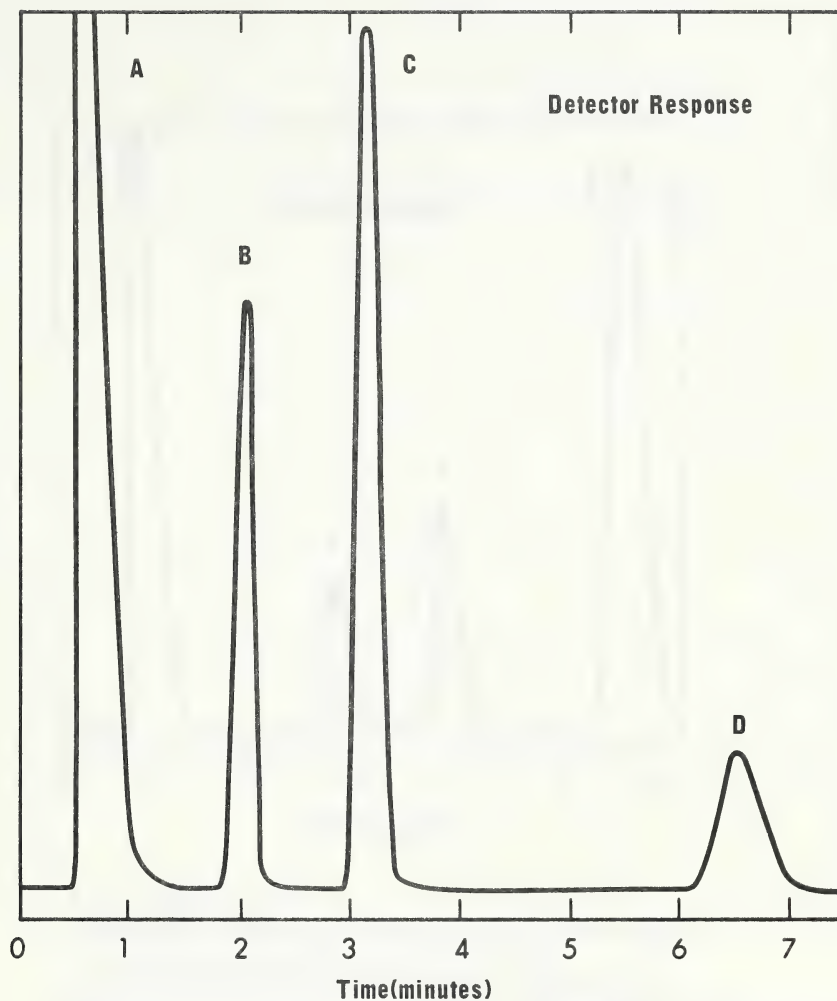


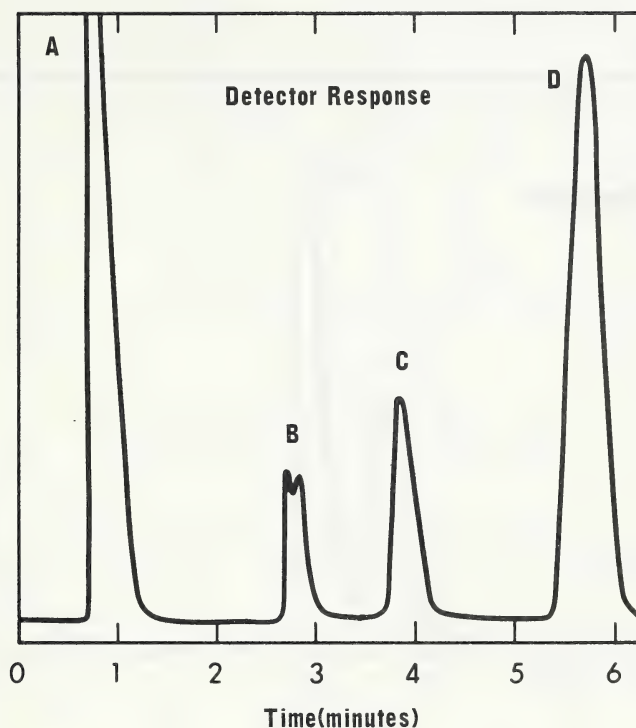
FIGURE 22b: CALIBRATION CURVE FOR NORETHYNODREL FROM THE NORETHNODREL AND MESTRANOL SOLUTION MIXTURES.



- A. Chloroform Solvent
B. Mestranol (Retention time 2.0 min.) *(Ratio, 3.78)
C. Cholestane (Retention time, 3.1 min.) *(Ratio, 5.39)
D. Chlormadinone Acetate (Retention time, 6.5 min.) *(Ratio, 1.00)

*All ratios are converted to the same attenuation for the diagram.

FIGURE 23: A TYPICAL CHROMATOGRAM OBTAINED FOR CHLORMADINONE ACETATE 6.0 ug./2 ul., MESTRANOL 3.0 ug./2 ul., AND CHOLESTANE 5.0 ug./2 ul.



A. Chloroform Solvent

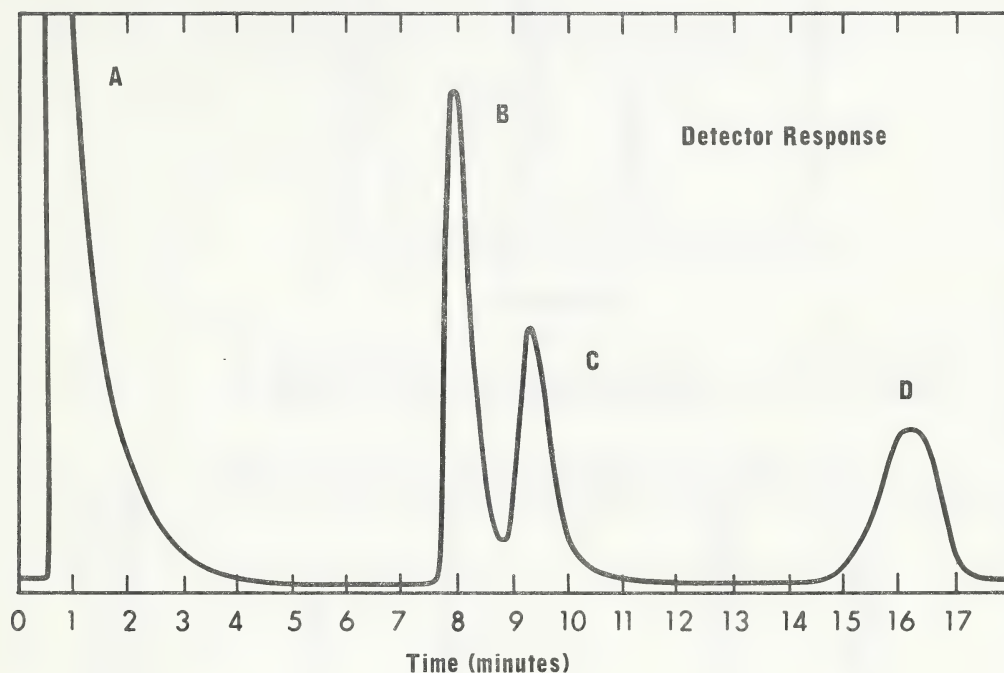
B. Ethynodiol Diacetate (Retention time for the first part of the split peak, 2.7 min.) *(Ratio, 1.00)

C. Mestranol (Retention time, 3.7 min.) *(Ratio 1.50)

D. Cholestane (Retention time, 5.7 min.) *(Ratio 3.51)

* All ratios are converted to the same attenuation for the diagram.

FIGURE 24: A TYPICAL CHROMATOGRAM OBTAINED FOR ETHYNODIOL DIACETATE 6.0 $\mu\text{g.}/2 \text{ ul.}$, MESTRANOL 3.0 $\mu\text{g.}/2 \text{ ul.}$, AND CHOLESTANE 5.0 $\mu\text{g.}/2 \text{ ul.}$



A. Chloroform Solvent

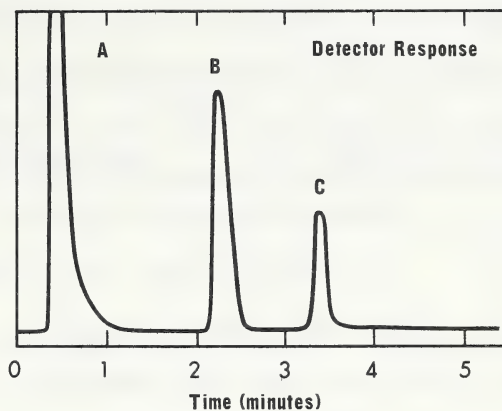
B. Norethynodrel (Retention time, 8.1 min.) *(Ratio, 3.05)

C. Mestranol (Retention time, 9.4 min.) *(Ratio, 1.60)

D. Cholestane (Retention time, 16.3 min.) *(Ratio 1.00)

*All ratios are converted to the same attenuation for the diagram.

FIGURE 25: A TYPICAL CHROMATOGRAM OBTAINED FOR NORETHYNODREL
12.0 ug./2 ul., MESTRANOL 6.0 ug./2 ul., AND CHOLESTANE 5.0 ug./2 ul.



- A. Chloroform Solvent
 B. Mestranol and Norethindrone overlap (Retention time, 2.2 min.)(Ratio, 1.90)
 C. Cholestane (Retention time, 3.2 min.)(Ratio, 1.00)

Proof of peak B being an overlapped Mestranol and Norethindrone peak

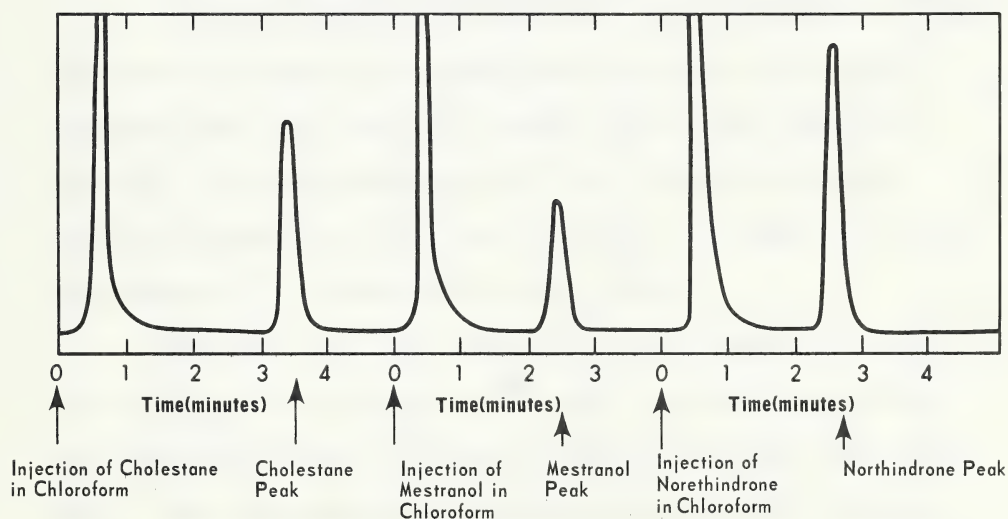


FIGURE 26: AN ILLUSTRATION OF THE RETENTION TIME, SHAPE OF PEAKS AND OVERLAPPING OF PEAKS OBTAINED FOR NORETHINDRONE 10.0 $\mu\text{g.}/2 \text{ ul.}$, MESTRANOL 5.0 $\mu\text{g.}/2 \text{ ul.}$, AND CHOLESTANE 5.0 $\mu\text{g.}/2 \text{ ul.}$

of height of steroid/height of cholestane was calculated, the concentration of steroid was read directly from the linear calibration curve. This concentration per known volume of solution was converted to concentration per tablet of oral contraceptive using the formula:

$$\text{Potency} = \frac{\text{Weight of one average tablet} \times \text{Concentration reading from the calibration curve}}{\text{Weight of powdered sample}}$$

Per cent recovery was calculated as:

$$\text{Per cent Recovery} = \frac{\text{Calculated potency per tablet}}{\text{Concentration per tablet declared on label}} \times 100$$

Table XII is a summary of the data obtained from the assay of the pharmaceutical dosage forms. A more detailed representation of the same analyses is found in Table XVII (Appendix).

In the gas-liquid chromatographic assay of Enovid-E[®] the chromatogram appeared uncomplicated showing only the three expected peaks for mestranol, norethynodrel, and cholestane. However, in some samples an extra very low and broad peak appeared between mestranol and cholestane, perhaps due to some decomposition or contamination of the sample.

When the percentage recoveries for mestranol and norethynodrel present in Enovid-E[®] were calculated, the former was found to be slightly high and the latter slightly low (see Table XII). This may possibly be due to one or more of the following reasons:

- (a) mestranol and norethynodrel peak very closely on the chromatogram and slightly overlap at their bases. After altering the column conditions in an attempt to facilitate separation of these steroids, cholestane showed a longer

TABLE XII

Assay of Pharmaceutical Dosage FormsA. Combination Tablets

<u>Trade Name</u>	<u>Quantity of Steroid Present (Manufacturer's Claim)</u>	<u>Trial</u>	<u>Estrogen Average</u>	<u>Trial</u>	<u>Progestogen Average</u>
1. Enovid-E [®]	Mestranol 0.1 mg. with Norethynodrel 2.5 mg.	1 104.9 2 110.3 3 116.4 4 112.5 5 113.3	111.5	1 83.2 2 82.2 3 91.4 4 90.0 5 88.9	87.1
2. Ovulen [®] 0.5 mg.	Mestranol 0.1 mg. with Ethinodiol Diacetate 0.5 mg.	1 137.5 2 133.8 3 136.7 4 138.2 5 135.3	136.3	1 165.8 2 211.6 3 211.5 4 204.1 5 193.1	197.2
<u>B. Sequential Tablets</u>					
1a. C-Quens [®]	Peach tablets: 0.08 mg. Mestranol with 2 mg. Chlormadinone Acetate	1 49.9 2 49.7 3 49.4 4 50.1 5 50.5		1 51.8 2 52.3 3 51.0 4 50.5 5 51.3	51.4
1b. C-Quens [®]	White tablets: 0.08 mg. Mestranol	1 50.7 2 49.8 3 49.8 4 49.3 5 50.8	50.1	49.9	
2. Ortho-Novum [®] SQ	White tablets: 0.08 mg. Mestranol	Using Ethinodiol Diacetate and Mestranol Standard Curve			
		1 77.4 2 123.9 3 83.0 4 67.8 5 53.8			81.2

retention time and appeared as a broader and shorter peak. Thus, in departing from the ideal narrow and sharp peak required for analytical measurement, an accurate assay became more difficult.

- (b) norethindrone and mestranol had the same retention time on the 3 per cent OV-1 column (see Figure 26). Substantial variation of both oven temperature and carrier gas flow rate was attempted to separate these two peaks but was unsuccessful. Evidence from the infrared spectrophotometric analysis indicated that some solutions of norethynodrel in chloroform, after a period of about 24 hours, were converted to norethindrone. A rise in temperature caused the same result in a shorter length of time. This could possibly be occurring in the Enovid-E[®] preparation upon GLC analysis, causing a higher estrogen recovery (average 111.5 per cent) and a lower progestogen recovery (average 87.1 per cent). In this instance, there would seem to be a partial conversion of norethynodrel to norethindrone, the norethindrone adding to the mestranol peak. This concurs with the IR analysis of Enovid-E[®] which gave an average progestogen recovery of 93.0 per cent. With Ovulen[®] 0.5 mg., the components mestranol and ethynodiol diacetate both gave results much higher than the manufacturer's claim, i.e., an average of 136.3 per cent for the estrogen and 197.2 per cent for the progestogen. Two possible causes of these high recoveries are the following:

- (a) ethynodiol diacetate appears as an overlapping doublet peak on the 3 per cent OV-1 column. In the majority of chromatograms

the first of the two peaks was the higher, but occasionally both peaks were of the same height or the second peak was higher than the first. In the preparation of the calibration curve for mestranol and ethynodiol diacetate this incongruity was minor and seldom appeared, but with the pharmaceutical trade preparation its appearance was more prevalent and resulted in varying peak height measurements.

- (b) another noted fact was the appearance of two additional peaks between the solvent and ethynodiol diacetate peaks. The infrared assay of Ovulen [®] 0.5 mg. also showed extra overlapping diluent peaks obscuring the ethynodiol diacetate and mestranol peaks.

C-Quens [®] was not assayed by infrared spectrophotometry because of a thick deposit on the inner portion of the solution cell, and the obtained spectra showed changes in absorption, perhaps indicating a chemical reaction. In the gas-liquid chromatographic assay of C-Quens [®] the attenuation was set at the lowest possible value to enable steroid peaks of measurable height to appear on the chromatogram. However, base line drift made accurate measurement difficult. Extra peaks also were apparent but it was difficult to determine whether these were merely due to base line distortions (background "noise") or due to tablet excipients.

In 1964 Schulz and Diaz reported the tablet excipients of a preparation containing 2.0 mg. chlormadinone acetate and 80 mcg. mestranol to be cornstarch, lactose and magnesium stearate. Subsequently, Schulz (1965) reported that the quantitation by GLC of mestranol when found in the presence of norethindrone or

chlormadinone acetate is impossible due to the partial decomposition of mestranol to the estrone methyl ether on a 1 per cent QF-1 column. This observation appears to agree with the assayed results given here using a 3 per cent OV-1 column, i.e., a very low recovery of mestranol (49.9 per cent). In addition, since both the estrogen and progestogen recoveries are approximately half of the manufacturer's claim, perhaps the method utilized did not result in quantitative extraction of the component steroids.

Since the estrogen of the anti-fertility preparations was in a concentration too low to be assayed by the infrared procedure, an effort was made to quantitatively assay the estrogen component of the sequential preparations C-Quens[®] and Ortho-Novum[®] SQ using GLC. Both of these products contained 0.08 mg. mestranol. The appearance of the chromatograms for the two preparations was very similar with regard to additional peaks other than mestranol. A major problem was the occurrence of a small peak near its base line which interfered with the quantitative measurement of mestranol. However, this did not occur in the chromatograms for pure mestranol. Clearly, the extra overlapping peak was either a tablet excipient or a decomposition product of the steroid. Schulz (1965) also reported that in formulations containing polyvinylpyrrolidone, which the trade preparations of C-Quens[®] and Ortho-Novum[®] SQ might contain, a complex is formed between mestranol and PVP making quantitative extraction of mestranol impossible.

In the gas-liquid chromatographic analysis of these anti-fertility preparations using the method developed here, all calculated percentage recoveries varied widely from the claimed potency. The

reasons for this differed but included the partial decomposition of steroids or the overlapping of peaks on the 3 per cent OV-1 column.

The problem might be resolved by the use of a more efficient extraction procedure, by the conversion of strongly polar functional groups to a more volatile derivative, and/or by the utilization of an integrating device to measure area under a peak rather than using peak height. If all these alternatives should fail, the careful choice of another column stationary phase may provide the answer.

SUMMARY AND CONCLUSIONS

The aim of the present study was to quantitatively assay the synthetic steroid components of the oral anti-fertility preparations. Through the developed infrared spectrophotometric and gas-liquid chromatographic analytical procedures it was found that:

A. Infrared Spectrophotometry

- (1) the absorption peak chosen for quantitative infrared analysis in each of the two estrogens and eight progestogens studied gave a linear and reproducible calibration curve when the steroid was analyzed in pure form.
- (2) all of the steroid powders appeared to be chemically stable in the selected chloroform solvent, with the exception of norethindrone when left in solution for about 24 hours. A rise in temperature caused the same result in a shorter length of time.
- (3) siliconization of glassware was necessary to prevent adsorption of the steroid samples onto their glass vessels. An initial calibration curve prepared from steroid solutions contained in non-siliconized glassware resulted in an extrapolated linear concentration-absorbance relationship which did not pass through the origin, but was slightly below. When the same procedure was repeated using siliconized glassware the extrapolated line passed through the origin.
- (4) in the analysis of the trade preparations, clear solutions obtained after centrifugation were directly analyzable through infrared spectrophotometry, while opaque solutions obtained even after prolonged centrifugation first required filtration through a Millipore membrane.

- (5) from the initial spectrum of a trade preparation it became apparent that the estrogen component was not sufficiently concentrated to give a clearly measurable absorption peak. Through the use of increased numbers of tablets the estrogen absorption became stronger. Unfortunately, diluent peaks also increased in intensity causing sufficient overlap to make estrogen measurement impossible.
- (6) of the 20 pharmaceutical dosage forms assayed through the developed infrared method, 10 of these gave progestogen percentage recoveries corresponding to the manufacturer's claim.
- (7) the majority of the estrogen-progestogen combinations found in commercial preparations gave a progestogen peak free of interference from diluent peaks. An exception was the combination of norethindrone and mestranol which included the trade names of Norinyl [®] 1 mg., 2 mg., Ortho-Novum [®] 1 mg., 2 mg., 5 mg., SQ, and Norquen [®]. In these samples the norethindrone 1660 cm^{-1} peak was completely obscured by a much stronger 1670 cm^{-1} diluent absorption. Infrared analysis of these preparations through the developed method was not possible.
- (8) Ovulen [®] 0.5 mg. did not give a clear ethynodiol diacetate peak although the product known as Ovulen [®] Three Week (1.0 mg.) did. The difference between the dosage forms could possibly be due to concentration. As more tablets were required for the assay of the former product, it appears likely that a larger quantity of diluent also dissolved in the

chloroform solution. Resolution of this problem may be obtained by a more efficient separation of steroids and tablet diluents.

- (9) the peach tablets of C-Quens [®], each containing 2 mg. chlormadinone acetate and 80 μ g. mestranol, were not analyzable through the developed infrared method. There appeared to be a chemical reaction occurring in the chloroform solution which progressively changed the resultant spectra with the passage of time.
- (10) the white tablets of Serial 28 [®], containing 1.0 mg. megestrol acetate and 0.1 mg. ethinyl estradiol, gave percentage recoveries lower than the claimed potency. In two attempts, one using a volume of 2.0 ml. and the other using a volume of 5.0 ml. of solution, the recovery rose from an average of 66.5 per cent to 73.0 per cent. A further increase in the number of tablets utilized was not attempted due to the high cost of the product. It seems likely, however, that an increase in volume to about 10 ml. would bring the percentage recovery up to the claimed potency.

B. Gas-liquid Chromatography

- (11) of the single estrogen and four progestogens studied on the 3 per cent OV-1 column, two estrogen-progestogen combinations, i.e., mestranol-chlormadinone acetate and mestranol-ethynodiol diacetate, gave linear and reproducible calibration curves when analyzed in pure form.
- (12) mestranol-norethynodrel gave a linear but non-reproducible

calibration curve when analyzed in pure form. The possible cause of non-reproducibility was the partial conversion of norethynodrel to norethindrone, as also noted in infrared spectrophotometry. Time would probably be a critical factor in the analysis of this particular combination.

- (13) pure mestranol and norethindrone had identical retention times, rendering the mestranol-norethindrone combination not suitable for analysis on the 3 per cent OV-1 column.
- (14) 5α -cholestane appeared to be a favorable choice as the internal standard in the quantitative GLC of the steroids studied.
- (15) using the method developed in the analysis of the selected anti-fertility preparations, all calculated percentage recoveries varied widely from the claimed potency.

Conclusions reached after the completion of this research project are the following:

- (a) infrared spectrophotometry is an efficient, accurate, precise, and reproducible quantitative method in the analysis of the majority of the anti-fertility progestogens.
- (b) gas-liquid chromatography is a more sensitive tool in quantitative measurement than infrared spectrophotometry, thus enabling estrogen measurement along with progestogen analysis. However, the developed analytical method was unacceptable for the trade preparations investigated. The problems encountered during analysis might be resolved through the use of a more efficient extraction procedure, by the conversion of strongly polar functional groups to a more volatile derivative, and/or by

the utilization of an integrating device to measure area under a peak rather than using peak height. If all these alternatives should fail, the careful choice of another column stationary phase may provide the answer. Since gas-liquid chromatography is a relatively new field, future experimental work in the assay of steroids clearly lies in this direction.

BIBLIOGRAPHY

- Abelson, D., and Bondy, P. K., 1955, Arch. Biochem., 57, 208.
- Baker, A. W., 1957, J. Phys. Chem., 61, 450.
- Bastow, R. A., 1967, J. Pharm. Pharmacol., 19, 41.
- Bender, A. E., and Wilson, A., 1947, Biochem. J., 41, 423.
- Beyer, W. F., 1968, J. Pharm. Sci., 57, 1415.
- Bird, H. L., Jr., Brickley, H. F., Comer, J. P., Hartsaw, P. E., and Johnson, M. L., 1963, Anal. Chem., 35, 346.
- Bliss, C. A., 1969, 'Pharmaceutical Chemistry' Volume 2, Editor L. G. Chatten, Marcel Dekker, New York and London, 1969, p. 655.
- Boughton, O. D., Bryant, R., Ludwig, W. J., and Timma, D. L., 1966, J. Pharm. Sci., 55, 951.
- 'The British Pharmacopoeia', 1968, The Pharmaceutical Press, 17 Bloomsbury Square, London, England, pp. 346, 394, 595, 664, 666, 667.
- Burchfield, H. P., and Storrs, E. E., 1962, 'Biochemical Applications of Gas Chromatography', Academic Press, New York and London, pp. 113, 517, 636.
- Bush, I. E., 1953, Fed. Proc., 12, 186.
- Bush, I. E., and Sandberg, A. A., 1953, J. Biol. Chem., 205, 783.
- Callow, R. K., and Kennard, O., 1961, J. Pharm. Pharmacol., 13, 723.
- Chen, P. S., 1959, Anal. Chem., 31, 292.
- Chissell, J. F., 1964, J. Pharm. Pharmacol., 16, 490.
- Clayton, R. B., 1962, Biochemistry, 1, 357.
- Colton, F. B., (G. D. Searle and Co.), 1955, U.S. 2,721,871.

- Colton, F. B., Nysted, L. N., Riegel, B., and Raymond, A. L., 1957, J. Amer. Chem. Soc., 79, 1123.
- Comer, J. P., Hartsaw, P., and Stevenson, C. E., 1968, J. Pharm. Sci., 57, 147.
- Corner, G. W., 1935, Amer. J. Physiol., 113, 238.
- Corner, G. W., and Allen, W. M., 1929, ibid., 88, 326.
- Corner, G. W., and Allen, W. M., 1936, Proc. Soc. Exp. Biol. Med., 34, 723.
- Cross, A. D., Landis, P. W., and Murphy, J. W., 1965, Steroids, 5, 655.
- Cullen, L. F., Rutgers, J. G., Lucchesi, P. A., Papariello, G. J., 1968, J. Pharm. Sci., 57, 1857.
- David, A., Fellowes, K. P., and Millson, D. R., 1959, J. Pharm. Pharmacol., 11, 491.
- Duggan, D. E., Bowman, R. L., Brodie, B. B., and Udenfriend, S., 1957, Arch. Biochem., 68, 1.
- Dyer, J. R., 1965, 'Applications of Absorption Spectroscopy of Organic Compounds', Prentice-Hall, Inc., Englewood Cliffs, N. J., p. 22.
- Edgren, R. A., Smith, H., Peterson, D. A. L., and Carter, D. L., 1963, Steroids, 2, 319.
- Eisenbrand, J., and Picher, H., 1939, Z. Phys. Chem., 260, 83.
- Ettre, L. S., 1967, 'The Practice of Gas Chromatography', Editors L. S. Ettre and A. Zlatkis, Interscience Publishers, New York, London, Sydney, 1967, p. 373.
- Fales, H. M., and Luukkainen, T., 1965, Anal. Chem., 37, 955.

- Gänshirt, H. G. , and Polderman, J. , 1964, J. Chromatogr. ,
16, 510.
- Garratt, D. C. , and Marshall, P. G. , 1954, J. Pharm. Pharmacol. ,
6, 950.
- Gibbs, H. D. , 1927, J. Biol. Chem. , 72, 649.
- Goodman, L. S. , and Gilman, A. , 1965, 'The Pharmacological Basis
of Therapeutics', Third Ed. , The Macmillan Company, New York,
N.Y. , p. 1540.
- Gornall, A.G. , and MacDonald, M. P. , 1953, J. Biol. Chem. ,
201, 279.
- Greenblatt, R. B. , 1956, J. Clin. Endocr. , 16, 869.
- Hara, S. , and Mibe, K. , 1967, Chem. Pharm. Bull. (Tokyo), 15,
1036.
- Hara, S. , and Mibe, K. , 1968, Anal. Chem. , 40, 1605.
- Hara, S. , Watabe, T. , Ike, Y. , and Ikekawa, N. , 1967, Chem.
Pharm. Bull. (Tokyo), 15, 1041.
- Hertz, R. , Waite, J. H. , and Thomas, L. B. , 1956, Proc. Soc.
Exp. Biol. Med. , 91, 418.
- Heusghem, C. , and Jehotte, J. M. , 1957, J. Pharm. Belg. , 12 ,
418; Chem. Abs. 52, 3259b.
- Higuchi, W. I. , Lau, P. K. , Higuchi, T. , and Shell, J. W. , 1963 ,
J. Pharm. Sci. , 52, 150.
- Hüttenrauch, R. , and Keiner, I. , 1965, Pharmazie , 20, 242.
- Iriarte, J. , Djerassi, C. , and Ringold, H. J. , 1959, J. Amer.
Chem. Soc. , 81, 436.
- Jenson, K. B. , 1952, Acta Pharmacol. (Kobenhavn), 8, 101.
- Jenson, K. B. , 1953, ibid. , 9, 66.

- Keay, G. R. , 1968, Analyst (London), 93, 28.
- Khoury, A. J. , and Cali, L. J. , 1967, J. Pharm. Sci. , 56, 1485.
- Kirchner, J. G. , Miller, J. M. , and Rice, R. G. , 1954, J. Agric. Fd. Chem. , 2, 1031.
- Klebe, J. F. , Finkbeiner, H. , and White, D. M. , 1966, J. Amer. Chem. Soc. , 88, 3390.
- Kober, S. , 1931, Biochem. Z. , 239 209.
- Kolthoff, I. M. , and Lingane, J. J. , 1952, 'Polarography', Volume II, Interscience, New York, N. Y. , 2nd Edition.
- Konii, S. , 1961, Yakugaku Zasshi , 81, 1016; Chem. Abs. 55, 27,775h.
- Kuhnert-Brandstätter, M. , Junger, E. , and Kofler, A. , 1965, Microchem. J. , 9, 105.
- Legrand, M. , Delaroff, V. , and Smolik, R. , 1958, J. Pharm. Pharmacol. , 10, 683.
- Liebermann, S. , 1952, J. Clin. Invest. , 31, 341.
- Linford, J. H. , 1952, Can. J. Med. Sci. , 30, 199.
- Linford, J. H. , and Paulson, O. B. , 1952, ibid. , 30, 213.
- Longecker, H. , 1961, Acta Endocr. , 37, 14.
- Luukkainen, T. , VandenHeuvel, W. J. A. , Haahti, E. O. A. , and Horning, E. C. , 1961, Biochim. Biophys. Acta , 52, 599.
- Matthews, J. S. , Pereda, A. L. , and Aguilera, A. , 1962, J. Chromatogr. , 9, 331.
- McKerns, K. W. , 1963, Biochem. Biophys. Acta , 69, 417.
- Mears, E. , 1964, Proc. Roy. Soc. Med. , 57, 204.
- Mesley, R. J. , 1966, Spectrochim. Acta , 22, 889.

- Mesley, R. J., and Johnson, C. A., 1965, J. Pharm. Pharmacol., 17, 329.
- Mitchell, F. L., and Davies, R. E., 1954, Biochem. J., 56, 690.
- Morton, R. A., and Stubbs, A. L., 1946, Analyst, 71, 348.
- Nakanishi, K., 1964, 'Infrared Absorption Spectroscopy', Holden-Day, Inc., San Francisco and Nankodo Company Limited, Tōkyo, p. 17.
- Page, J. E., 1957, Chem. Industr. (London), 58.
- Pheasant, R., 1950, J. Amer. Chem. Soc., 72, 4303.
- Pincus, G., 1956, Acta Endocr. Suppl. No. 28, 18.
- Pincus, G., Chang, M. C., Hafez, E. S. E., Zarrow, M. X., and Merrill, A., 1956, Science, 124, 890.
- Pincus, G., Chang, M. C., Zarrow, M. X., Hafez, E. S. E., and Merrill, A. 1956, Endocrinology, 59, 695.
- Pincus, G., Rock, J., Garcia, C. R., Rice-Wray, E., Paniagua, M., and Rodriguez, I., 1958, Amer. J. Obstet. Gynec., 75, 1333.
- Prestegard, E. L., 1967, Amer. J. Med. Techn., 33, 437.
- Rao, C. N. R., 1963, 'Chemical Applications of Infrared Spectroscopy', Academic Press, New York and London, p. 406.
- Rehm, C. R., and Smith, J. B., 1960, J. Amer. Pharm. Ass., Sci. Ed., 49, 386.
- Ringold, H. J., Batres, E., Bowers, A., Edwards, J., and Zderic, J., 1959, J. Amer. Chem. Soc., 81, 3485.
- Roberts, G., 1957, Anal. Chem., 29, 911.
- Roberts, H. R., and Florey, K., 1962, J. Pharm. Sci., 51, 794.
- Roberts, H. R., Siino, M. R., 1963, ibid., 52, 370.

- Rock, J., Garcia, C. R., and Pincus, G., 1957, Recent Progr. Hormone Res., 13, 323.
- Rock, J., Pincus, G., and Garcia, C. R., 1956, Science, 124, 891.
- Röpke, H., and Neudert, W., 1959, Z. Analyt. Chem., 170, 78.
- Schmulowitz, J. J., and Wylie, H. B., 1935, J. Lab. Clin. Med., 21, 210.
- Schulz, E. P., 1965, J. Pharm. Sci., 54, 144.
- Schulz, E. P., and Diaz, M. A., 1964, ibid., 53, 1115.
- Shroff, A. P., and Grodsky, J., 1967, ibid., 56, 460.
- Shroff, A. P., and Huettemann, R. E., 1967, ibid., 56, 654.
- Sjöstrom, E., and Nykanen, L., 1957, J. Amer. Pharm. Ass., Sci. Ed., 46, 321.
- Sjövall, J., Meloni, C. R., and Turner, D. A., 1961, J. Lipid Res., 2, 317.
- Slaunwhite, W. R., Jr., Engel, L. L., Scott, I. F., and Ham, C. L., 1953, J. Biol. Chem., 201, 615.
- Smakula, E., Gori, A., and Wotiz, H., 1957, Spectrochim. Acta, 9, 346.
- Smith, L. L., and Foell, T., 1959, Anal. Chem., 31, 102.
- Sweat, M. L., 1954, ibid., 26, 1964.
- Swyer, G. I. M., and Little, V., 1962, Proc. Roy. Soc. Med., 55, 861.
- Talbot, N. B., Wolfe, J. K., MacLachlan, E. A., Karush, F., and Butler, A. M., 1940, J. Biol. Chem., 134, 319.
- Talmage, J. M., Penner, M. H., and Geller, M., 1965, J. Pharm. Sci., 54, 1194.

- Templeton, R. J., Arnett, W. A., and Jakovljevic, I. M., 1968, ibid., 57, 1168.
- Tishler, F., Sheth, P. B., Giaimo, M. B., and Mader, W. J., 1962, ibid., 5, 1175.
- Touchstone, J. C., and Murawec, T., 1960, Anal. Chem., 32, 822.
- Tsilifonis, D. C., and Chafetz, L., 1967, J. Pharm. Sci., 56, 625.
- Tyler, E. T., and Olson, H. J., 1959, J. Amer. Med. Wom. Ass., 169, 1843.
- Umberger, E. J., 1955, Anal. Chem., 27, 768.
- 'The United States Pharmacopeia', 17th rev., 1965, Mack Publishing Co., Easton, Pa., p. 245.
- Urganyi, T., and Rehm, C. R., 1966, J. Pharm. Sci., 55, 501.
- VandenHeuvel, W. J. A., and Horning, E. C., 1963, Biochim. Biophys. Acta, 74, 560.
- VandenHeuvel, W. J. A., Sjövall, J., and Horning, E. C., 1961, ibid., 48, 594.
- VandenHeuvel, W. J. A., Sweeley, C. C., and Horning, E. C., 1960, J. Amer. Chem. Soc., 82, 3481.
- Venning, E. H., Evelyn, K. A., Harkness, E. V., and Browne, J. S. L., 1937, J. Biol. Chem., 120, 225.
- Wells, D., Katzung, B., and Meyers, F. H., 1961, J. Pharm. Pharmacol., 13, 389.
- Wotiz, H. H., and Martin, H. F., 1961, J. Biol. Chem., 236, 1312.

APPENDIX

TABLE XIII

Data for the Determination of Calibration Curves

<u>Concentration</u>	<u>Absorbance</u>			
	<u>Trial 1</u>	<u>Trial 2</u>	<u>Trial 3</u>	<u>Average*</u>
<u>Estrogens</u>				
1. Ethinyl Estradiol				
3.0 mg./ml.	0.03	0.03	0.03	0.03
5.0 mg./ml.	0.06	0.06	0.06	0.06
10.0 mg./ml.	0.12	0.13	0.12	0.12
16.0 mg./ml.	0.19	0.20	0.19	0.19
20.0 mg./ml.	0.24	0.24	0.24	0.24
2. Mestranol				
1.5 mg./ml.	0.02	0.02	0.02	0.02
3.0 mg./ml.	0.06	0.05	0.05	0.05
6.0 mg./ml.	0.11	0.11	0.11	0.11
11.0 mg./ml.	0.21	0.20	0.21	0.21
15.0 mg./ml.	0.27	0.27	0.26	0.27
19.0 mg./ml.	0.34	0.34	0.35	0.34
<u>Progestogens</u>				
3. Chlormadinone Acetate				
1.25 mg./ml. \simeq 1.3 mg./ml.	0.07	0.07	0.07	0.07
2.5 mg./ml.	0.15	0.14	0.14	0.14
5.0 mg./ml.	0.28	0.28	0.29	0.28
8.0 mg./ml.	0.47	0.44	0.45	0.45
10.0 mg./ml.	0.55	0.54	0.54	0.54

Continued . . .

TABLE XIII - Continued

<u>Concentration</u>	<u>Absorbance</u>			
	<u>Trial 1</u>	<u>Trial 2</u>	<u>Trial 3</u>	<u>Average *</u>
4. Dimethisterone				
1.05 mg./ml. \approx 1.1 mg./ml.	0.08	0.07	0.07	0.08
2.1 mg./ml.	0.16	0.15	0.15	0.15
2.7 mg./ml.	0.19	0.19	0.19	0.19
3.6 mg./ml.	0.28	0.25	0.26	0.26
4.2 mg./ml.	0.31	0.29	0.29	0.30
4.8 mg./ml.	0.35	0.34	0.35	0.35
5. Ethynodiol Diacetate				
0.25 mg./ml. \approx 0.3 mg./ml.	0.02	0.01	0.02	0.02
0.5 mg./ml.	0.03	0.03	0.03	0.03
1.0 mg./ml.	0.07	0.07	0.07	0.07
1.5 mg./ml.	0.11	0.11	0.10	0.11
2.0 mg./ml.	0.15	0.15	0.14	0.14
2.5 mg./ml.	0.19	0.18	0.17	0.18
3.0 mg./ml.	0.22	0.21	0.21	0.21
4.0 mg./ml.	0.29	0.28	0.28	0.28
5.0 mg./ml.	0.36	0.36	0.36	0.36
6.0 mg./ml.	0.43	0.43	0.41	0.42
6. Megestrol Acetate				
1.0 mg./ml.	0.06	0.07	0.07	0.07
2.0 mg./ml.	0.14	0.13	0.14	0.14
3.0 mg./ml.	0.16	0.20	0.21	0.20
4.0 mg./ml.	0.26	0.26	0.27	0.26
5.0 mg./ml.	0.31	0.34	0.31	0.32

Continued

TABLE XIII - Continued

<u>Concentration</u>	<u>Absorbance</u>			
	<u>Trial 1</u>	<u>Trial 2</u>	<u>Trial 3</u>	<u>Average*</u>
7. Norethindrone				
0.5 mg./ml.	0.06	0.05	0.05	0.05
1.0 mg./ml.	0.09	0.09	0.09	0.09
2.5 mg./ml.	0.23	0.24	0.24	0.24
3.5 mg./ml.	0.33	0.32	0.33	0.33
5.0 mg./ml.	0.47	0.45	0.47	0.46
8. Norethindrone Acetate				
1.25 mg./ml. \approx 1.3 mg./ml.	0.11	0.11	0.11	0.11
2.5 mg./ml.	0.20	0.20	0.20	0.20
5.0 mg./ml.	0.39	0.41	0.39	0.40
6.4 mg./ml.	0.50	0.49	0.49	0.50
8.0 mg./ml.	0.62	0.59	0.60	0.60
9. Norethynodrel				
1.0 mg./ml.	0.06	0.06	0.06	0.06
2.5 mg./ml.	0.14	0.15	0.15	0.15
3.5 mg./ml.	0.20	0.21	0.20	0.21
5.0 mg./ml.	0.29	0.28	0.29	0.29
7.0 mg./ml.	0.39	0.40	0.39	0.39
10. d-Norgestrel : (as <u>dl</u> -racemate)				
1.0 mg./ml.	0.10	0.10	0.09	0.10
2.0 mg./ml.	0.18	0.19	0.18	0.18
3.0 mg./ml.	0.27	0.28	0.27	0.27
4.0 mg./ml.	0.35	0.36	0.35	0.35
5.0 mg./ml.	0.45	0.47	0.42	0.45

* Estimate of uncertainty in the last significant figure is ± 5 .

TABLE XIV

Assay of Pharmaceutical Dosage Forms

Trade Name	Steroid Analyzed	Absorbance		Analyzed Steroid Concentration per Tablet (mg.)		Per cent Recovery		Average \pm One Standard Deviation			
		Trial		Trial		Trial					
A. Combination Tablets											
1. Enovid [®] 5 mg.	Norethynodrel 5.0 mg.	1	0.216	0.215	1	4.85	4.82	1	97.0	96.4	98.6 \pm 1.9
		2	0.232	0.225	2	4.94	4.80	2	98.8	96.0	
		3	0.225	0.231	3	4.91	5.05	3	98.2	101.0	
		4	0.231	0.231	4	5.03	5.03	4	100.6	100.6	
		5	0.203	0.196	5	5.02	4.85	5	100.4	97.0	
2. Enovid [®] 10 mg.	Norethynodrel 9.85 mg.	1	0.229	0.226	1	10.06	9.91	1	102.1	100.6	99.5 \pm 2.5
		2	0.233	0.226	2	10.13	9.83	2	102.8	99.8	
		3	0.226	0.231	3	9.87	10.07	3	100.2	102.2	
		4	0.221	0.221	4	9.47	9.47	4	96.1	96.1	
		5	0.218	0.223	5	9.53	9.73	5	96.8	98.8	
3. Enovid-E [®] Norethynodrel 2.5 mg.	Norethynodrel 2.5 mg.	1	0.158	0.160	1	2.33	2.34	1	93.2	93.6	93.0 \pm 2.3
		2	0.154	0.155	2	2.27	2.30	2	90.8	92.0	
		3	0.165	0.154	3	2.41	2.27	3	96.4	90.8	
		4	0.164	0.168	4	2.36	2.42	4	94.4	96.8	
		5	0.159	0.158	5	2.28	2.27	5	91.2	90.8	
4a. Norlestrin [®] Norethindrone Acetate 1.0 mg. (per 2.0 ml. solution)	Norethindrone Acetate 1.0 mg. (per 2.0 ml. solution)	1	0.224	0.225	1	0.73	0.73	1	73.0	73.0	76.6 \pm 2.5
		2	0.238	0.238	2	0.77	0.77	2	77.0	77.0	
		3	0.244	0.245	3	0.79	0.79	3	79.0	79.0	
		4	0.254	0.254	4	0.79	0.79	4	79.0	79.0	
		5	0.245	0.245	5	0.75	0.75	5	75.0	75.0	
4b. Norlestrin [®] Norethindrone Acetate 1.0 mg. (per 5.0 ml. solution)	Norethindrone Acetate 1.0 mg. (per 5.0 ml. solution)	1	0.147	0.147	1	0.90	0.90	1	90.0	90.0	90.8 \pm 2.5
		2	0.140	0.135	2	0.90	0.87	2	90.0	87.0	
		3	0.147	0.150	3	0.94	0.96	3	94.0	96.0	
		4	0.141	0.140	4	0.91	0.90	4	91.0	90.0	
		5	0.149	0.149	5	0.90	0.90	5	90.0	90.0	

Continued . . .

TABLE XIV - Continued

Trade Name	Steroid Analyzed	Absorbance		Analyzed Steroid Concentration per Tablet (mg.)		Per cent Recovery		Average \pm One Standard Deviation			
		Trial		Trial		Trial					
5. Norlestrin [®] 2.5 mg.	Norethindrone Acetate 2.5 mg.	1	0.237	0.237	1	2.34	2.34	1	93.6	93.6	92.6 \pm 1.2
		2	0.239	0.244	2	2.33	2.37	2	93.2	94.8	
		3	0.241	0.239	3	2.31	2.30	3	92.4	92.0	
		4	0.241	0.239	4	2.29	2.28	4	91.6	91.2	
		5	0.238	0.238	5	2.29	2.29	5	91.6	91.6	
6. Ovral [®]	d-Norgestrel (as dl-racemate) 0.25 mg.	1	0.166	0.166	1	0.26	0.26	1	104.0	104.0	104.0 \pm 2.3
		2	0.168	0.170	2	0.26	0.26	2	104.0	104.0	
		3	0.165	0.167	3	0.25	0.25	3	100.0	100.0	
		4	0.178	0.177	4	0.27	0.27	4	108.0	108.0	
		5	0.175	0.174	5	0.26	0.26	5	104.0	104.0	
7. Ovulen [®] Three Week	Ethinodiol Diacetate 1.0 mg.	1	0.090	0.086	1	1.05	1.01	1	105.0	101.0	101.6 \pm 2.2
		2	0.084	0.081	2	1.00	0.98	2	100.0	98.0	
		3	0.088	0.088	3	1.04	1.04	3	104.0	104.0	
		4	0.085	0.082	4	1.02	1.00	4	102.0	100.0	
		5	0.079	0.082	5	1.00	1.02	5	100.0	102.0	

TABLE XV

Assay of Pharmaceutical Dosage Forms

B. Sequential Tablets		Trade Name	Steroid Analyzed	Absorbance		Analyzed Steroid Concentration per Tablet (mg.)		Per cent Recovery		Average \pm One Standard Deviation	
				Trial		Trial		Trial			
1. Oracon [®]	Dimethisterone 25 mg.	1	0.176	0.174	1	23.5	23.2	1	94.1	93.0	95.0 \pm 2.9
		2	0.188	0.190	2	24.9	25.1	2	99.6	100.3	
		3	0.177	0.178	3	23.7	23.8	3	94.9	95.2	
		4	0.174	0.177	4	22.8	23.3	4	91.2	93.0	
		5	0.187	0.191	5	23.5	23.8	5	94.0	95.1	
2. Ovex [®]	Megestrol Acetate 5.0 mg.	1	0.180	0.179	1	4.67	4.65	1	93.4	93.0	93.8 \pm 3.1
		2	0.194	0.188	2	4.84	4.73	2	96.8	94.6	
		3	0.175	0.171	3	4.53	4.45	3	90.6	89.0	
		4	0.188	0.186	4	4.61	4.61	4	92.2	92.2	
		5	0.105	0.105	5	4.90	4.90	5	98.0	98.0	
3. Secrovin [®]	Dimethisterone 25 mg.	1	0.215	0.215	1	24.9	24.9	1	99.6	99.6	101.1 \pm 1.6
		2	0.207	0.210	2	25.5	26.0	2	102.1	103.9	
		3	0.176	0.181	3	25.1	25.8	3	100.5	103.4	
		4	0.177	0.181	4	24.8	25.4	4	99.0	101.4	
		5	0.190	0.187	5	25.3	25.0	5	101.3	100.2	
4a. Serial 28 [®]	Megestrol Acetate 1.0 mg. (per 2.0 ml. solution)	1	0.089	0.090	1	0.67	0.68	1	67.0	68.0	66.5 \pm 2.0
		2	0.092	0.090	2	0.69	0.68	2	69.0	68.0	
		3	0.089	0.090	3	0.67	0.68	3	67.0	68.0	
		4	0.086	0.085	4	0.64	0.63	4	64.0	63.0	
		5	0.087	0.082	5	0.66	0.65	5	66.0	65.0	
4b. Serial 28 [®]	Megestrol Acetate 1.0 mg. (per 5.0 ml. solution)	1	0.102	0.099	1	0.74	0.72	1	74.0	72.0	73.0

TABLE XVI

Data for the Preparation of Calibration Curves

A. Chlormadinone Acetate and Mestranol

Solution	Concentration	($\mu\text{g.}/2\mu\text{l.}$)	Peak Height (cms.)	Attenuation	Height of steroid in cms.	
					Height of Cholestane	Height of Cholestane in cms.
1 _B	Chlormadinone Acetate	10.0	16.40	200	0.285	
	Mestranol	5.0	13.10	1000	1.139	
	Cholestane	5.0	11.50	1000	-	
2 _B	Chlormadinone Acetate	8.0	14.10	200	0.237	
	Mestranol	4.0	10.92	1000	0.918	
	Cholestane	5.0	11.90	1000	-	
3 _B	Chlormadinone Acetate	6.0	13.10	200	0.186	
	Mestranol	3.0	19.80	500	0.702	
	Cholestane	5.0	14.11	1000	-	
4 _B	Chlormadinone Acetate	4.0	10.00	100	0.110	
	Mestranol	2.0	19.42	200	0.428	
	Cholestane	5.0	9.08	1000	-	
5 _B	Chlormadinone Acetate	2.0	6.08	100	0.055	
	Mestranol	1.0	12.33	200	0.222	
	Cholestane	5.0	11.13	1000	-	
6 _B	Chlormadinone Acetate	1.0	3.00	100	0.025	
	Mestranol	0.5	12.50	100	0.103	
	Cholestane	5.0	12.12	1000	-	

Continued . . .

TABLE XVI - Continued

B. Ethynodiol Diacetate and Mestranol

Solution	Concentration	$(\mu\text{g.}/2\mu\text{l.})$	Peak Height (cms.)	Attenuation	Height of steroid in cms.	
					Height of Cholestane in cms.	Height of steroid in cms.
1 B	Ethynodiol Diacetate	10.0	14.50	200		0.787 (this point is no longer linear)
	Mestranol	5.0	7.99	500		1.075
	Cholestane	5.0	18.42	200		-
2 B	Ethynodiol Diacetate	8.0	8.05	200		0.541
	Mestranol	4.0	13.10	200		0.879
	Cholestane	5.0	14.90	200		-
3 B	Ethynodiol Diacetate	6.0	5.51	200		0.416
	Mestranol	3.0	9.02	200		0.681
	Cholestane	5.0	13.25	200		-
4 B	Ethynodiol Diacetate	4.0	10.19	100		0.285
	Mestranol	2.0	7.64	200		0.428
	Cholestane	5.0	17.87	200		-
5 B	Ethynodiol Diacetate	2.0	2.86	100		0.144
	Mestranol	1.0	4.40	100		0.222
	Cholestane	5.0	9.93	200		-
6 B	Ethynodiol Diacetate	1.0	1.60	100		0.064
	Mestranol	0.5	2.60	100		0.104
	Cholestane	5.0	12.55	200		-

Continued . . .

C. Norethynodrel and Mestranol

(i) First Determination			Peak Height ($\mu\text{g.}/2\mu\text{l.}$) (cms.)	Attenuation	Height of steroid in cms.
Solution	Concentration				Height of Cholestane in cms.
A	Norethynodrel	24.0	10.45	500	5.806
	Mestranol	12.0	5.75	500	3.194
	Cholestane	5.0	9.00	100	-
1 B	Norethynodrel	20.0	10.35	500	4.929
	Mestranol	10.0	5.63	500	2.681
	Cholestane	5.0	10.50	100	-
2 B	Norethynodrel	16.0	19.75	200	4.114
	Mestranol	8.0	10.50	200	2.188
	Cholestane	5.0	9.60	100	-
3 B	Norethynodrel	12.0	16.30	200	3.052
	Mestranol	6.0	8.50	200	1.592
	Cholestane	5.0	10.68	100	-
4 B	Norethynodrel	8.0	11.25	200	2.093
	Mestranol	4.0	5.80	200	1.079
	Cholestane	5.0	10.75	100	-
5 B	Norethynodrel	4.0	8.50	100	0.998
	Mestranol	2.0	4.40	100	0.516
	Cholestane	5.0	8.52	100	-
6 B	Norethynodrel	2.0	8.40	50	0.455
	Mestranol	1.0	4.45	50	0.241
	Cholestane	5.0	18.45	50	-
7 B	Norethynodrel	1.0	10.82	20	0.262
	Mestranol	0.5	5.75	20	0.139
	Cholestane	5.0	16.50	50	-

Continued . . .

TABLE XVI - Continued

(ii) Second Determination

Solution	Concentration	$(\mu\text{g.}/2\mu\text{l.})$	Peak Height (cms.)	Attenuation	Height of steroid in cms.		Average of (i) First and (ii) Second Determinations
					Height of Cholestane	Height of Cholestane	
A	Norethynodrel	24.0	14.60	500	5.062		5.434
	Mestranol	12.0	8.71	500	3.020		3.107
	Cholestane	5.0	14.42	100	-		-
1B	Norethynodrel	20.0	21.50	200	4.175		4.552
	Mestranol	10.0	12.75	200	2.476		2.579
	Cholestane	5.0	10.30	100	-		-
2B	Norethynodrel	16.0	18.50	200	3.246		3.680
	Mestranol	8.0	11.42	200	2.004		2.096
	Cholestane	5.0	11.40	100	-		-
3B	Norethynodrel	12.0	15.70	200	2.361		2.707
	Mestranol	6.0	9.90	200	1.492		1.542
	Cholestane	5.0	13.30	100	-		-
4B	Norethynodrel	8.0	18.60	100	1.646		1.870
	Mestranol	4.0	11.75	100	1.040		1.060
	Cholestane	5.0	11.30	100	-		-
5B	Norethynodrel	4.0	9.40	100	0.838		0.918
	Mestranol	2.0	5.50	100	0.490		0.503
	Cholestane	5.0	11.22	100	-		-
6B	Norethynodrel	2.0	3.55	100	0.414		0.435
	Mestranol	1.0	2.08	100	0.242		0.242
	Cholestane	5.0	8.58	100	-		-
7B	Norethynodrel	1.0	8.00	20	0.199		0.231
	Mestranol	0.5	4.85	20	0.120		0.130
	Cholestane	5.0	16.10	50	-		-

TABLE XVII

Assay of Pharmaceutical Dosage Forms

A. Combination Tablets

	Trial	Height of Steroid Peak (cms.)	Attenuation	Height of steroid	Per cent Recovery
1. Enovid-E [®] Mestranol 0.1 mg., Norethynodrel 2.5 mg.					
Estrogen	1	2.32	100	0.286	104.9
	2	2.53	100	0.275	110.3
	3	2.70	100	0.294	116.4
	4	2.75	100	0.275	112.5
	5	3.00	100	0.284	113.3
Average Estrogen Recovery = 111.5					
Progestogen	1	7.81	500	4.821	83.2
	2	7.97	500	4.332	82.2
	3	8.61	500	4.684	91.4
	4	9.39	500	4.690	90.0
	5	9.79	500	4.640	88.9
Average Progestogen Recovery = 87.1					
Cholestane	1	8.10	100	-	-
	2	9.20	100	-	-
	3	9.19	100	-	-
	4	10.01	100	-	-
	5	10.55	100	-	-
Average Cholestane Recovery = 100.0					
2. Ovulen [®] 0.5 mg. Mestranol 0.1 mg., Ethynodiol Diacetate 0.5 mg.					
Estrogen	1	4.10	100	0.159	137.5
	2	4.30	100	0.152	133.8
	3	3.90	100	0.157	136.7
	4	4.30	100	0.160	138.2
	5	4.19	100	0.152	135.3
Average Estrogen Recovery = 136.3					

Continued . . .

TABLE XVII - Continued

	Trial	Height of Steroid Peak (cms.)	Attenuation	Height of steroid	Per cent
				Height of Cholestane	Recovery
Progesterogen	1	3.75	200	0.291	165.8
	2	10.47	100	0.371	211.6
	3	9.20	100	0.369	211.5
	4	9.60	100	0.356	204.1
	5	9.07	100	0.329	193.1
Cholestane	1	12.90	200	-	-
	2	14.10	200	-	-
	3	12.45	200	-	-
	4	13.48	200	-	-
	5	13.80	200	-	-
B. Sequential Tablets					
1. C-Quens® 15 white tablets: 0.08 mg Mestranol					
5 peach tablets: 2 mg. Chlormadinone Acetate with 0.08 mg. Mestranol					
(a) Peach Tablets					
Estrogen	1	1.72	5	0.024	49.9
	2	3.15	2	0.024	49.7
	3	2.55	2	0.023	49.4
	4	2.60	2	0.021	50.1
	5	3.15	2	0.024	50.5
Progesterogen	1	3.40	10	0.093	51.8
	2	2.50	10	0.095	52.3
	3	2.00	10	0.089	51.0
	4	4.28	5	0.087	50.5
	5	4.78	5	0.092	51.3
Average Progesterogen Recovery = 197.2					
Average Estrogen Recovery = 49.9					
Average Progesterogen Recovery = 51.4					

Continued . . .

TABLE XVII - Continued

Trial	Height of Steroid Peak (cms.)	Attenuation	Height of steroid		Per cent Recovery
			Height of Cholestane	Recovery	
Cholestane					
1	18.20	20	-	-	-
2	13.10	20	-	-	-
3	11.21	20	-	-	-
4	12.36	20	-	-	-
5	13.06	20	-	-	-
(b) White Tablets					
Estrogen					
1	2.85	200	0.096	50.7	Average Estrogen Recovery = 50.1
2	1.65	200	0.088	49.8	
3	4.28	200	0.105	49.8	
4	3.22	200	0.083	49.3	
5	6.00	200	0.099	50.8	
Cholestane					
1	11.93	500	-	-	-
2	7.51	500	-	-	-
3	16.35	500	-	-	-
4	15.57	500	-	-	-
5	24.30	500	-	-	-
2. Ortho-Novum® SQ 14 white tablets: 0.08 Mestranol Using Ethynodiol Diacetate and Mestranol Standard Curve					
Estrogen					
1	3.10	200	0.250	77.4	Average Estrogen Recovery = 81.2
2	5.58	100	0.358	123.9	
3	5.45	100	0.296	83.0	
4	3.60	100	0.198	67.8	
5	2.82	100	0.183	53.8	
Cholestane					
1	12.42	200	-	-	-
2	15.60	100	-	-	-
3	18.40	100	-	-	-
4	18.15	100	-	-	-
5	15.45	100	-	-	-

